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Title:
The role of insulin like growth factor II in pre-adipocyte regulation

The role of insulin like growth factor II in pre-adipocyte regulation

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2019

A dissertation submitted to the University of Bristol in accordance with the requirements for the award of degree of Doctor of Philosophy in the Faculty of Health Sciences, School of Clinical Sciences

Word count:44,319

Abstract

The IGF system has an important role in growth and development. IGF-II is a recognised fetal growth promoter. However, its physiological role in post-natal life remains uncertain, although it is maintained in the circulation at a substantially high level throughout life, exceeding IGF-I levels. IGF-II has been strongly linked to body weight and obesity in genetic studies and more recent evidence suggests a metabolic role. We examined fat depot differences in the actions of IGF-II in respect to growth and metabolism. We used an established adipocyte, cell culture system of matched pairs of visceral and subcutaneous fat biopsies from 20 normal weight children undergoing routine surgery for non-malignant, non-septic conditions. Multiple cell culture techniques were used to examine the role of IGF-II; cell counting and tritiated thymidine incorporation were used to assess the effect on proliferation. Oil Red O staining was used to assess preadipocyte differentiation, western blotting and reverse transcription polymerase chain reaction techniques were employed to assess the levels of adipogenesis markers and levels of the receptors and insulin receptor isoforms. Metabolic function was assessed by radioactive glucose uptake assay. We speculated a specific effect of IGF-II on visceral adipocytes in relation to the differential distribution of insulin receptor isoforms between visceral and subcutaneous fat depots. Initial characterisation of receptor levels indicated that visceral preadipocytes have higher levels of insulin receptor isoform A than those of subcutaneous preadipocytes. With differentiation, insulin receptor isoform B was increased; however, visceral adipocytes still maintained a higher expression of IR-A than that of subcutaneous adipocytes. A difference in IGF-IIR/M6P and IGF-IR was also seen between the fat depots. IGF-II promoted preadipocyte proliferation, and when preadipocytes were differentiated for 14 days in the presence or absence of IGF-II it prompted preadipocyte differentiation in subcutaneous preadipocytes but showed an opposing effect restricting visceral preadipocyte differentiation, which was confirmed by reductions in differentiation markers: PPARγ, adiponectin and in triglyceride staining. Additionally, IGF-II reduced mRNA expression of the insulin receptor in adipocytes,

and downregulated IR-A and GLUT4 abundance and corresponding glucose uptake in visceral adipocytes.

To further elaborate whether IGF-II plasma levels can be used as a potential biomarker of fat distribution, data from a lifecourse cohort, ALSPAC, were used to correlate early IGF-II levels with fat distribution measured by DXA scans during puberty. Interestingly, and in broad agreement with our cell culture data, cord blood IGF-II was negatively correlated with total mass and, in particular, trunk fat mass and showed a positive association with subcutaneous fat mass measured in the legs and arms, with adjustment of age and sex confounders but no association with lean, fat-free mass. In conclusion, IGF-II is a regulator of preadipocyte differentiation and metabolism by acting in a depot-specific manner as a differential modulator of fat accumulation favouring less visceral fat deposition in children, as well as being a suggested early-life predictor of later fat distribution.

Acknowledgements

I wish to express my deepest gratitude to my supervisors — Prof. Jeffery Holly, Dr. Claire Perks, and Prof. Julian Hamilton-Shield — for their expert supervision and continuous encouragement throughout my PhD time. You were always there for advice, answering questions and providing help when needed. Thank you for prompting me to become a better researcher; without you this work would never have been completed.

I would like to acknowledge Imam Abdulrahman Bin Faisal University for funding this work. I would also like to thank Urology Surgeons Mr. M Shalaby, Mr. G Nicholls, and Mr. M Woodward, as well as the theatre staff at the Bristol Royal Hospital for Children, for their help in collecting the biopsies. I acknowledge the General Surgery and Urology waiting list coordinators, Matthew Price and Edward Appleton, for their help in the recruitment process. Furthermore, I would like to extend my acknowledgment to the NIHR Bristol Nutrition Unit Team for their warm welcome and hospitability, Prof. Andy Ness for his help and supervision, and Dr. Sam Leary for the statistical consultation in the ALSPAC study.

I would like to thank all of the IMEG research group members — Kalina, Hanna, Athba, Rehanna, Moses, Caroline, Ahmad, Hannah and Reham — for creating such a friendly and supportive work environment.

Last but not least, I would like to thank my beloved Mom for her endless love, encouragement and prayers; my dearest husband, Suliman, for being my rock through good and rough times (thank you for your devoted care and for making this opportunity possible); and my little son, Dawood, for being my joy and motivation. My gratitude extends to my lovely sisters and my brother, who were always cheering for me, and to all of my family for their love and support. I am also thankful to my friend Noura for her continuous kind support. I am appreciative to you all for sharing this journey with me.

Dedication

This thesis is dedicated to the memory of my beloved father, ***Nasser I. Alfares****, who sadly passed away in 2017. To him I will be forever grateful, not only for being an amazing father but also for being my best friend. You will always be missed, Dad.*

Author’s Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: ..... DATE: .....

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List of Abbreviations

3T3-L1	Mouse (Swiss albino) fibroblast cell line
ADD-1	Adipocyte determination and differentiation factor 1
AKT	Protein kinase B
ALS	Acid-labile subunit
ALSPAC	Avon Longitudinal Study of Parents and Children
AMP	AMP-activated protein
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
aP2	Adipocyte-specific fatty acid binding protein-2
APO	Apotransferrin
APS	Ammonium persulfate
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BCA	Bicinchoninic acid
BMI	Body mass index
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CDS	Cell dissociation solution
cDNA	Complementary Deoxyribonucleic acid
C/EBP	CCAAT/enhancer binding protein
cm	Centimetre
CO_2	Carbon dioxide
CREG	Cellular repressor of E1A-stimulated gene
CTCF	Transcriptional repressor
DXA	Dual-energy X-ray absorptiometry
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DOG	2-deoxyglucose
DPM	Disintegrations per minute

ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ERK1/2	Extracellular signal regulated kinase 1/2
ELISA	Enzyme linked immunosorbent assay
FASN	Fatty acid synthase
FBS	Fetal bovine serum
FFA	Free fatty acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT 1	Glucose transporter 1
GLUT 4	Glucose transporter 4
GH	Growth hormone
GM	Growth medium
HBSS	Hanks' balanced salt solution
HCl	Hydrochloric acid
HDL	High-density lipoprotein (HDL) cholesterol
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HiFBS	Heat-inactivated fetal bovine serum
HRP	Horseradish peroxidase-conjugated
IBMX	3-isobutyl-1-methylxanthine
ICR	Imprinting control region
IGF-I	Insulin-like growth factor-I
IGF-II	Insulin-like growth factor-II
IGF-IR	Insulin-like growth factor-I receptor
IGF-IIR/M6P	Insulin-like growth factor-II receptor / mannose-6-phosphate receptor
IGFBPs	Insulin-like growth factor binding proteins
IL-6	Interleukin-6
IR	Insulin receptor
IR-A	Insulin receptor isoform A
IR-B	Insulin receptor isoform B
IRS	Insulin receptor substrate
KCL	Potassium chloride

kDa	Kilo Dalton
Kg	Kilogram
KRP	Krebs Ringer phosphate
LOI	Loss of imprinting
LSD	Least significant difference
M	Molar
MAPK	Mitogen-activated protein kinases
mg	Milligram
$MgSO_4$	Magnesium sulfate
ml	Millilitre
mRNA	Messenger Ribonucleic acid
MW	Molecular weight
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaH_2PO_4	Monosodium dihydrogen orthophosphate
NaOH	Sodium hydroxide
NCS	Newborn calf serum
NHS	Normal human serum
NS	Not significant
n/µ/p	nano/ micro/ pico
OD	Optical density
ORO	Oil red O stain
PAPP-A	Pregnancy-associated plasma protein
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol-4, 5-bisphosphate
PIP3	Phosphatidylinositol-3, 4, 5-triphosphate
PKC	Protein kinase C
PPAR-α	Peroxisome proliferator-activated receptor-α
PPAR-γ	Peroxisome proliferator-activated receptor-γ
PREF-1	Preadipocyte factor-1
PTEN	Phosphatase and tensin homolog
PS	Penicillin and streptomycin
QRT-PCR	Quantitative real time polymerase chain reaction

RCF	Relative centrifugal force
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RNase	Ribonuclease
RTK	Receptor tyrosine kinase
RXR	Retinoid X receptor
SCFA	Short chain fatty acids
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate- polyacrylamide gel electrophoresis
SE	Standard error
SEM	Standard error of the mean
SFM	Serum free medium
Shc	Src homology and collagen protein
SOC	Standard occupational classification
SREBP-1	Sterol regulatory element-binding protein-1
T_3	3,3',5-Triiodo-L-Thyronine
TBST	Tris buffered saline tween-20
TCA	Trichloroacetic acid
TE	Trypsin EDTA solution
TEMED	N, N, N, N- Tetramethylethylenediamine
TGF-β	Transforming growth factor-beta
TGN	Trans-Golgi network
Triton X-100	(t-octyl phenoxypoly-ethoxyethanol)
Tris base	(Tris (hydroxymethyl)aminomethane)
TNF-α	Tumour necrosis factor α
TTI	Tritiated thymidine incorporation
Tween 20	Polyoxyethylenesorbitan monolaurate
UCP1	Uncoupling protein1
V	Volts
VEGF	Vascular endothelial growth factor
WAT	White adipose tissue

WB	Western blotting
WHO	World health organization

Manuscripts arising from this work

Alfares, M. N., Perks, C. M., Hamilton-Shield, J. P., & Holly, J. M. (2018). Insulin-like Growth Factor II (IGF-II) in Adipocyte Regulation: Depot-Specific Actions Suggest a Potential Role Limiting Excess Visceral Adiposity. *American Journal of Physiology-Endocrinology and Metabolism*: 2018 Jul 24. doi: 10.1152/ajpendo. 00409.2017. PMID: 30040480. ***Published***

Alfares, M.N., Leary, S. D., Ness, A. R., Perks, C. M., Holly, J. M & Hamilton-Shield, J. P. Correlation between prepubertal IGF-II and post-pubertal fat distribution: findings from the Avon Longitudinal Study of Parents and Children (ALSPAC)- ***Manuscript under preparation***

Academic communication and abstracts submitted related to this work

May 2018 - 25th European Congress on Obesity, Vienna, Austria (poster presentation)

March 2017- Gordon Research Conferences: IGF& insulin System in Physiology & Disease, Ventura, USA (poster presentation)

December 2016- SOCS academic day Bristol, UK (oral presentation)

October 2016- Bristol Nutrition NIHR Biomedical Research Unit Scientific Meeting, Bristol, UK (oral presentation)

October 2016- International Conference on Diabetes and Metabolism, Seoul, Korea (oral presentation)

May 2016- SOCS postgraduate seminar talk, Bristol, UK

February 2016- The 9th Saudi Students Conference, Birmingham, UK (poster presentation)

December 2015- SOCS academic day Bristol, UK (poster presentation)

May 2015- SOCS postgraduate seminar talk, Bristol, UK

September 2014- SOCS postgraduate seminar talk, Bristol, UK

Awards/grants related to this work

- **September 2018-**Saudi Arabian excelling students reward
- **March 2017**-Bristol university alumni funding £500
- **October 2016**-Outstanding abstract travel grant, International Conference on Diabetes and Metabolism, $1000
- **December 2016**-SOCS academic day first prize winner for oral presentation £50

Chapter 1 : General introduction

1.1 Obesity

Obesity is defined by the World Health Organization (WHO) as an abnormal or excessive fat accumulation that may impair health (WHO 2000). Individuals are classified as overweight or obese in terms of their body mass index, or BMI. BMI is calculated by dividing a person's weight in kilograms by his or her height in metres squared [kg/m^2], with a BMI over 25 classified as overweight, while a BMI of 30 or above is considered obese. The BMI classification is shown in Table 1-1.

Table 1-1 Body mass index (BMI) classification *(WHO 2000)*

Classification	BMI (kg/m^2)
Underweight	< 18.5
Normal range	18.5 – 24.9
Overweight	≥ 25
Pre-obese	25 – 29.9
Obese I	30 – 34.9
Obese II	35 – 39.9
Obese III	≥ 40

Obesity is a global problem, with 39% of men and 40% of women aged 18 or above considered overweight worldwide and a total of more than half a billion adults worldwide being obese. A minimum of 2.8 million people die yearly as a result of being overweight or obese. In fact, 65% of the world's population live in countries where more people die of being overweight and obese than being underweight. Overweight and obesity are responsible for 44% of the diabetes burden, 23% of the ischaemic heart disease burden and between 7% and 41% of certain cancer burdens (WHO 2016).

The worldwide prevalence of obesity has nearly doubled between 1980 and 2008. In 2008, 10% of men and 14% of women in the world were obese in comparison with 5% of men and 8% of women in 1980. The highest prevalence in the world was in the United States, where 38.2% of both sexes were obese. The lowest percentage was in South East Asia, where around 3% of both sexes were obese (OECD 2017). In the United Kingdom, 26% were classified as obese, and more than 600 thousand were admitted to the National Health Service *(NHS)* hospitals in relation to obesity (PAS 2018).

1.1.2 Childhood obesity

Obesity is classified somewhat differently in children, with BMI calculated according to their age and sex. If a child's BMI is at or above the 85th percentile for his or her age, then he or she is at risk of becoming overweight. If a child's BMI is at or above the 95th percentile, the child is classified as obese (Ghosh 2014). The epidemic of childhood obesity has increased in the past four decades, with a surge in recent years. Furthermore, this increase in prevalence is not exclusive to higher income countries but also occurs in middle- and low-income counties as illustrated in Figure 1-1.

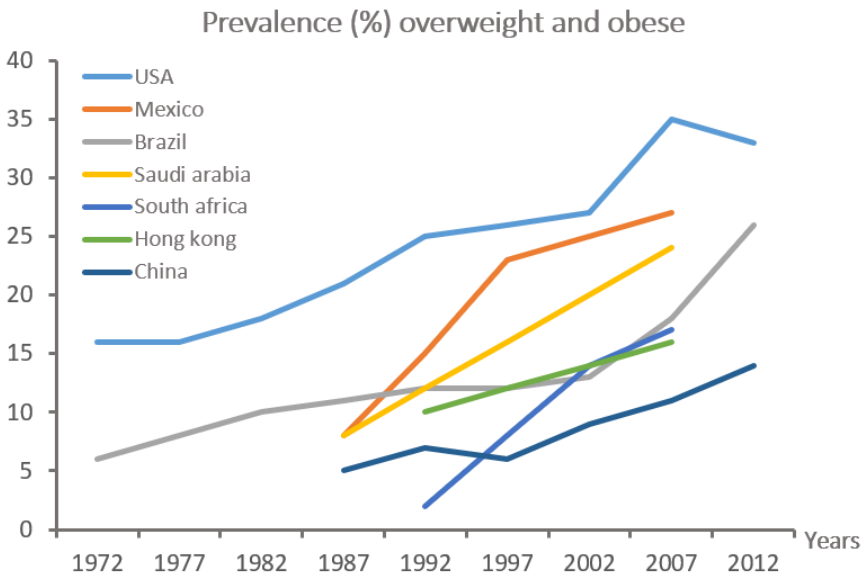


Figure 1-1 Prevalence trends for childhood overweight and obesity in the USA and eight low-income and middle-income countries. (*adapted from (Lobstein, Jackson-Leach et al. 2015))*

More than 41 million children worldwide under the age of 5 are overweight or obese (WHO 2016). In the US, the obesity prevalence has more than doubled in children between 1980 and 2010, with more than one third of the children and adolescents being overweight or obese in 2010 (Taber, Chriqui et al. 2011). In the UK, the 2015/16 National Child Measurement Programme (NCMP) showed that obesity or overweight prevalence among 4–5-year olds (reception) was 20% and among 10–11-year olds (year 6) was 33%. Childhood obesity is of particular concern as 25% of obese adults were overweight as children and if overweight begins before 8 years of age, obesity in adulthood is likely to be more severe (Freedman, Khan et al. 2005). In addition to the increase of obesity risk in adulthood, childhood obesity might be associated with long term health manifestation regardless of adult body weight (Must, Jacques et al. 1992). This

highlights the importance of early periods of growth monitoring and body weight related interventions, as they may have a huge impact on health status later in life.

1.1.3 Consequences of childhood obesity

Adult obesity-associated diseases are now becoming a paediatric problem; obese children are at a higher risk of developing related systemic and psychological problems (Dietz 2004, Lo, Chandra et al. 2014). The medical consequences of childhood obesity include dyslipidaemia, hypertension and increased cardiovascular disease risks (Stabouli, Kotsis et al. 2005). Increasing rates of type 2 diabetes mellitus were noted in American ethnic minorities in association with increased obesity rates (Rocchini 2002). Development of glucose intolerance (Wabitsch, Hauner et al. 1994), non-alcoholic fatty liver disease (Schwimmer, Deutsch et al. 2006) and cholelithiasis (Friesen and Roberts 1989) have also increased. In addition to the medical impact, psychosocial consequences have been observed including increased rates of anxiety, depression and weight-related social problems associated with bullying and low self-esteem (Puder and Munsch 2010).

1.2 The adipose tissue

1.2.1 Adipose tissue types

Adipose tissue is a specialised tissue that functions as the major storage site for fat and is found in mammals in two different forms: white and brown adipose tissue. They are composed of connective tissue, vascular and inflammatory cells and mainly white or brown adipocytes. The adipocytes in white adipose tissue (WAT) contain large

unilocular lipid droplets and in addition to its fat storage role, WAT is considered an active endocrine organ that regulates many major functions in the body, such as insulin sensitivity and lipid metabolism, by its ability to secrete multiple hormones, like leptin, angiotensinogen, adipsin, adiponectin and resistin that play an important role in physiological and metabolic functions (Trayhurn and Beattie 2001, Galic S 2010). WAT stores excess energy as triglycerides and releases the energy in the form of free fatty acids. It is the main type of adipose tissue found in adult humans as it distributed throughout the body in subcutaneous regions, surrounding visceral organs and extremities (Nedergaard 2008).

By contrast, brown adipose tissue (BAT) is found mostly in rodents, small mammals and in human infants. It is histologically distinct from WAT, as it is composed of multiloculated adipocytes that contain large numbers of mitochondria accounting for their brown colour (Nedergaard 2004). The importance of brown adipocytes lies in their ability to change stored chemical energy into heat, which occurs through the expression of uncoupling protein-1 (UCP1) found in the inner mitochondrial membrane. This protein permits dissipation of the mitochondrial proton gradient which is used to promote the production of ATP (Himms-Hagen 1990). For many years, BAT was thought to be absent in human adults, but recent fluorodeoxyglucose positron emission tomography studies of normal humans have identified regions of high glucose uptake that represent metabolically active tissue (Cypess, Lehman et al. 2009) mainly traced in the upper parts of the human body, particularly the supraclavicular and the neck regions and some in the paravertebral, para-aortic, mediastinal and suprarenal areas (Cypess, White et al. 2013).

A new adipocyte cell was recently added to the white and brown adipocytes, known as beige adipocytes or inducible ‘brown-like’ adipocytes. These cells are clusters of

UCP1-producing adipocytes with a heat generating ability, but develop in WAT due to different stimulation, such as agonists of the β-adrenergic receptor or peroxisome proliferator-activated receptor γ (PPARγ) (Vitali, Murano et al. 2012). This is considered a fundamental difference from brown adipocytes as they express high levels of UCP1 under basal or unstimulated conditions (Klaus, Ely et al. 1995). The beige adipocytes have a multilocular lipid droplet morphology and a high mitochondrial content, but whether they serve different functions in comparison to brown adipocytes is still to be determined (Wu, Boström et al. 2012). The adipose tissue types are shown in Figure 1-2.

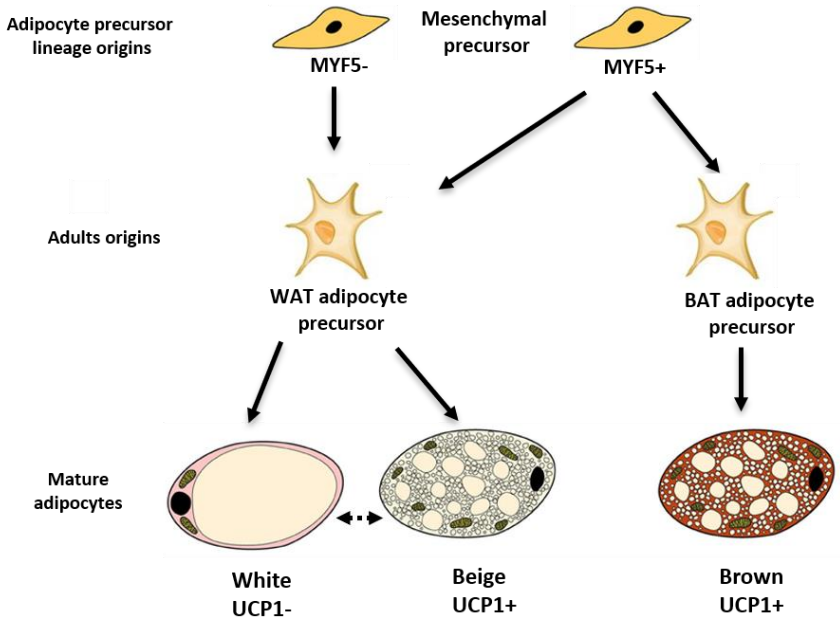


Figure 1-2 Origins of white, beige and brown adipocytes *(adapted from (Peirce, Carobbio et al. 2014))*

Brown adipose tissue originates from myogenic factor 5 (MYF5+) expressing cell lineage and is formed by $UCP1^{+}$ brown adipocytes, while white adipose tissue originates from non-myogenic expressing lineage and is formed by $UCP1^{-}$ white adipocytes and $UCP1^{+}$ beige adipocytes. The expansion of adipose tissue is due to proliferation and differentiation of adipocyte precursors which originate from mesenchymal precursor cells.

1.2.2 Molecular regulation of adipocyte differentiation

As indicated in the previous section (1.2.1), the adipocytes originate from multipotent mesenchymal precursor cells that can differentiate into mature cells of multiple mesenchymal tissues including fat and bone (Zuk, Zhu et al. 2002). The preadipocytes can become dormant or be processed to become differentiated adipocytes. For the differentiation phase, preadipocytes withdraw from the cell cycle, which usually occurs through contact inhibition, however cell-to-cell contact might not be an absolute requirement for growth arrest as 3T3-F44A cells were able to differentiate in suspension culture without contact inhibition (Hayashi, Nixon et al. 1981). After the growth arrest stage, the committed cells undergo clonal amplification, which is achieved by at least one round of cell division and DNA replication (Fajas 2003). Following the clonal expansion phase and with the appropriate combination of mitogenic and adipogenic signals, the cells will continue to differentiation. The preadipocytes will go through multiple biochemical and morphological changes leading to the accumulation of triglycerides in the vesicles that is associated with cytoskeletal remodelling and changes in the level and type of extracellular matrix (ECM). A fusion of visceral triglycerides will occur towards the end of the terminal differentiation phase, leading to the formation of the round mature adipocyte appearance (MacDougald and Lane 1995).

The regulation of preadipocyte differentiation occurs at the genetic level, leading to an increase in the levels of many transcription factors, including peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer binding protein (C/EBP), as well as increased levels of multiple lipid metabolism proteins,

such as fatty acid synthase (FASN), lipoprotein lipase (LPL), GLUT4, stearyl CoA and angiotensinogen (Rosen, Walkey et al. 2000).

1.2.2.1 Transcription factors PPARγ, C/EBPα, ADD-1/SREBP-1 and Pref-1

The C/EBP transcriptional factors C/EBPβ and C/EBPδ are involved in the early stages of adipogenic induction, followed by PPARγ and C/EBPα, which act cooperatively to activate one another and are involved in the growth arrest phase required for differentiation (Mandrup and Lane 1997). Furthermore, the promoter region of PPAR has binding sites for C/EBP (Mandrup and Lane 1997, Michalopoulos and DeFrances 1997). PPARγ activation induces the exit from the cell cycle and PPARγ is considered the most specific transcriptional factor for adipocyte differentiation (Grégoire, de Broux et al. 1992). The adipocyte determination and differentiation factor-1/sterol regulatory element-binding protein-1 (ADD-1/SREBP-1) is also expressed early in the process and stimulates the expression of many genes needed for lipogenesis *in vivo* (Ericsson, Jackson et al. 1997). ADD-1/SREBP-1 in combination with C/EBPβ and C/EBPδ induce the expression and/or activity of PPARγ, the controller of adipocyte differentiation (Fajas, Fruchart et al. 1998, Saladin, Fajas et al. 1999).

In contrast to these stimulatory transcription factors, preadipocyte factor 1 (Pref-1) acts as an inhibitor of adipocyte differentiation. Pref-1 is highly expressed in preadipocytes and its expression is decreased with differentiation. Pref-1 functions to maintain the preadipocyte phenotype, possibly by interacting with the extracellular matrix (ECM) (Smas and Sul 1993, Sul 2009). It was also reported to have an inhibitory effect on adipogenesis *in vivo* (Wang, Kim et al. 2006).

In the terminal differentiation phase, adipocytes increase *de novo* lipogenesis and become insulin-sensitive, which is associated with an increase in lipid metabolism

enzyme transcription and protein levels including glycerol-3-phosphate dehydrogenase and fatty acid synthase as well as other non-lipid metabolism related genes, like adipocyte-specific fatty acid binding protein (aP2). The stages of preadipocyte differentiation are demonstrated in Figure 1-3.

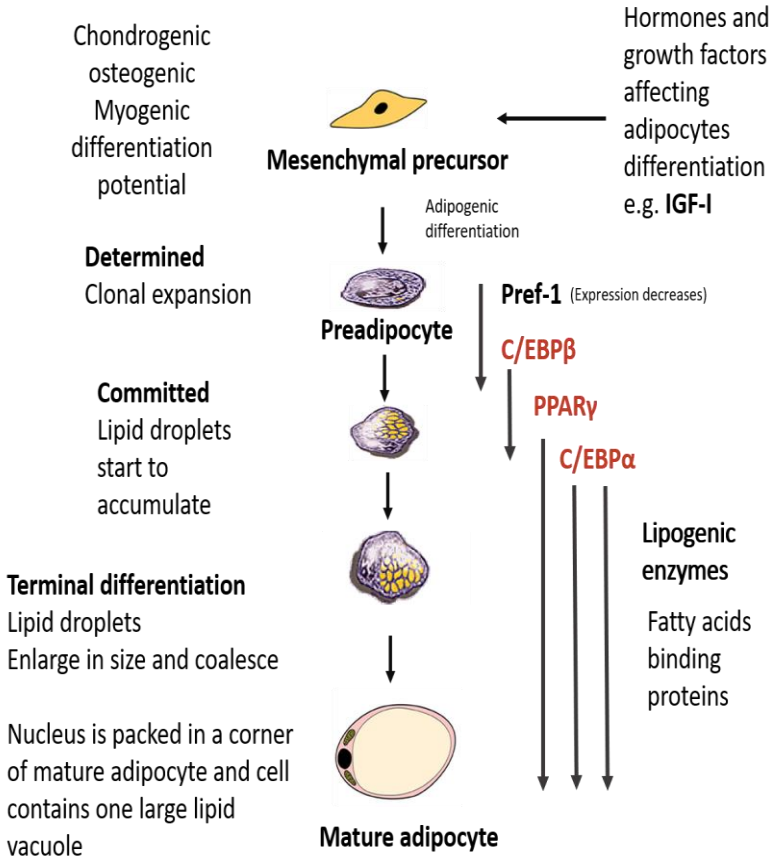


Figure 1-3 Illustration of the stages of preadipocyte differentiation *(adapted from (Chen, Roether et al. 2008))*

The multipotent stem cell has the ability to differentiate to adipocytes, myoblasts, chondroblasts and osteoblasts. When exposed to the appropriate adipogenic environment these stem cells will give rise to preadipocytes, which undergo clonal expansion phase and reach terminal differentiation. The transcriptional regulation associated with this process is shown on the right, the arrows indicate the approximate duration. *Abbreviations:* IGF-I, insulin-like factor-1; pref-1, preadipocyte factor 1; C/EBP, CCAAT/enhancer binding protein; PPARγ, peroxisome proliferator-activated receptor γ.

1.2.2.2 Cytoskeletal remodelling

The process of adipogenesis leads to dramatic morphological and cytoskeletal changes. Interestingly, these cytoskeletal rearrangements facilitate insulin signalling, as they provide support for key signalling components such as insulin receptor substrate (IRS), following Tyr-phosphorylation IRS acts as an anchor for other downstream molecules including SH2 (Src homology 2 domain) and p85 regulatory subunit of PI3K (Whitehead, Clark et al. 2000). Activation PI3K pathway lead to phosphorylation of Akt which is involved in GLUT4 glucose uptake (Taguchi and White 2008), and PKC family members which regulates actin cytoskeleton (Jacinto, Loewith et al. 2004). Maintaining the microtubule network arrangements are needed for the movement of GLUT4 vesicles from the intracellular compartments to the plasma membrane (Kanzaki and Pessin 2001). The changes in ECM level and type are seen with differentiation, including alteration in the basement membrane and the reticular fibre network, leading to differences in cellular adhesion properties, and remodelling of cell components allowing reorganisation and adipocyte gene expression (Gregoire, Smas et al. 1998).

1.2.3 Adipose tissue fat depots

Fat develops in many anatomical locations including subcutaneous, mesenteric, gluteal, femoral and intra-abdominal. The intra-abdominal fat is classified into visceral or intraperitoneal/perinephric adipose tissue and is found inside the peritoneal cavity. Subcutaneous adipose tissue lays anteriorly to the abdomen wall or back muscles. This classification is important because metabolic risks are found to be related more to body fat distribution rather than total body fat (Wajchenberg 2000). In

addition, differences in multiple internal characteristics were reported between the fat depots, including differences in the composition, endocrine secretions, hormonal responses and metabolic function. Figure 1-4 shows the anatomical distribution of subcutaneous and visceral fat.

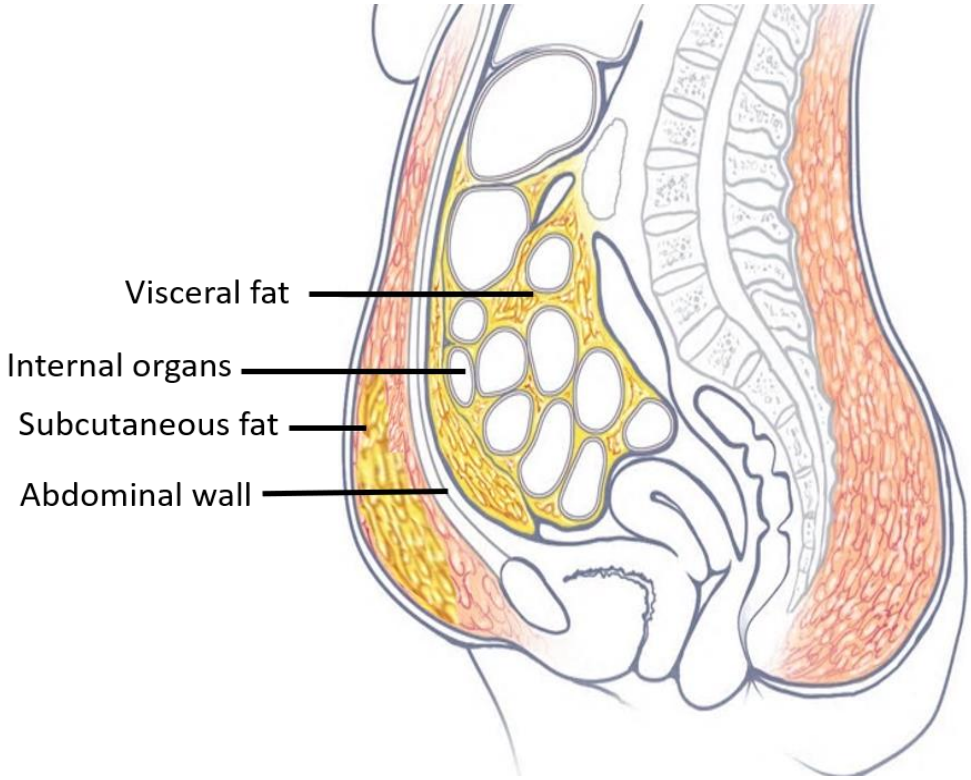


Figure 1-4 illustration of abdominal visceral and subcutaneous fat distribution

(adapted from www.mayoclinic.org).

Vertical cross section showing visceral adiposity as the fat surrounded by the visceral cavity and subcutaneous adiposity defined as the fat outside the visceral cavity.

1.2.3.1 Anatomical and developmental differences

Subcutaneous fat accounts for most of the body fat. The widespread distribution of subcutaneous fat has systemic venous drainage, whereas the internal location of visceral fat maintains direct drainage via the portal vein, facilitating direct access of

secreted FFAs and adipokines by visceral cells to the liver (Mårin, Andersson et al. 1992, Wajchenberg 2000).

Visceral and subcutaneous adipocytes can differ in their characteristics from early development, where the adipocyte precursor cells from subcutaneous fat are easier to differentiate, which may help to explain why subcutaneous fat expansion occurs mainly by hyperplasia in comparison to visceral fat that expands predominantly by hypertrophy of existing cells. The pattern of gene expression in subcutaneous cells has many similarities with the gene expression of brown fat in comparison with visceral adipocytes which have a different genetic expression profile (Macotela, Emanuelli et al. 2012).

Visceral and subcutaneous fat have separate parasympathetic innervation and visceral fat has an increased sympathetic supply, which may have a role in influencing fat storage in adipocytes (Bartness 2002).

1.2.3.2 Molecular and cellular differences

Multiple differences between visceral and subcutaneous cells in terms of receptor distribution and cellular signalling have been reported. Visceral cells have higher levels of glucocorticoid (Lundholm, Rebuffe-Scrive et al. 1985), androgenic (Freedland 2004) and adrenergic receptors (Hellmer, Marcus et al. 1992) than subcutaneous cells, which leads to an increase in catecholamine related lipolysis in visceral fat (Imbeault, Couillard et al. 2000). Instead, oestrogen receptors are more dominant in subcutaneous fat, with a higher density in females leading to more gluteal-femoral accumulation of fat in comparison to males (Pedersen, Hansen et al. 1996).

Furthermore, fat-depot differences in hormonal secretion have been noted. Visceral adipocytes are able to secrete more adiponectin (Motoshima, Wu et al. 2002) and angiotensinogen (Dusserre, Moulin et al. 2000) than subcutaneous fat. In addition,

pro-inflammatory cytokines, like TNF-α and IL-6, are higher in visceral cells (Lemieux, Pascot et al. 2001, Weisberg, McCann et al. 2003), whereas subcutaneous cells show more leptin production than visceral cells (Wajchenberg 2000).

These molecular differences increase the association between metabolic risks and visceral fat because of the accumulation of inflammatory macrophages and pathologic tissue expansion with a positive energy balance leading to rapid enlargement of existing adipocytes (hypertrophy) with poor vascularisation, which may be associated with fibrosis and an increased level of inflammatory cytokines. This form of expansion may further progress to cause ectopic fat accumulation in different body locations, such as the liver, pancreas and skeletal muscles (Stefan, Kantartzis et al. 2008, 2011).

1.2.3.3. Metabolic fat-depot differences

Insulin stimulated glucose uptake is higher in visceral adipocytes than in subcutaneous adipocytes in children (Grohmann, Sabin et al. 2005) and in adults (Lundgren, Burén et al. 2004). In contrast, insulin inhibition of lipolysis is decreased in visceral adipocytes relative to subcutaneous adipocytes (Zierath, Livingston et al. 1998). Visceral cells have increased lipolytic activity. The mechanism whereby visceral adipocytes are more insulin-sensitive for glucose uptake but less insulin-sensitive for inhibition of lipolysis has not been fully elucidated.

The clinical significance of these structural and metabolic differences between the fat depots shows that increased visceral obesity is associated with a higher metabolic risk (Rexrode, Carey et al. 1998) as well as increased risk of coronary heart disease (Yudkin, Kumari et al. 2000), stroke (Amato, Giordano et al. 2010), diabetes and metabolic syndrome (Lemieux and Despres 1994), thereby affecting individual life expectancy. Indeed, abdominal obesity is associated with higher mortality rates regardless of the BMI level (Zhang, Shu et al. 2007).

1.3 The insulin-like growth factor system

The insulin-like growth factor (IGF) system is a complex network essential for regulation of cell growth, proliferation and survival. It is important in embryonic and postnatal development as the system affects almost all the body organ systems. IGFs were identified in the early 1950s as somatomedins, however, when their insulin-like actions were discovered later, the term *insulin-like growth factors* was later introduced in 1976 (Rinderknecht and Humbel 1976).

The IGF system is composed of two soluble ligand peptide hormones IGF-I and IGF-II, which have high structural similarity to insulin. Multiple transmembrane receptors exist, the IGF-I receptor, the insulin receptor (IR), the IGF-II receptor and hybrid IR/IGF-IR. There are also six circulating high affinity IGF binding proteins (IGFBP-1 to 6) that also regulate the system (Rechler 1993). The majority of circulating IGFs (90%) are bound in complexes consisting of IGF-I or IGF-II and IGFBP-3 (to a lesser extent IGFBP-5) and the 85 kDa acid-labile subunit (ALS), which is a glycoprotein synthesised by the liver (Rajaram, Baylink et al. 1997). These complexes lead to more stabilisation and elongation of the half-life of the IGFs to 16–24 h. Around 10% of IGFs are found circulating as binary complexes with IGFBPs, which cross the capillary endothelium to act as a pericellular reservoir of IGFs, with a half-life of 20–30 minutes. The remaining less than 1% is free in the circulation; this unbound form has a very short half-life like insulin of a few minutes (Baxter 2014). The free bioactive hormone binds and activates a receptor tyrosine kinase (RTK) receptor, which recruits intracellular substrate proteins leading to biological effects (Braun, Bitton-Worms et al. 2011). The half-life of IGFs in the circulation is illustrated in Figure 1-5.

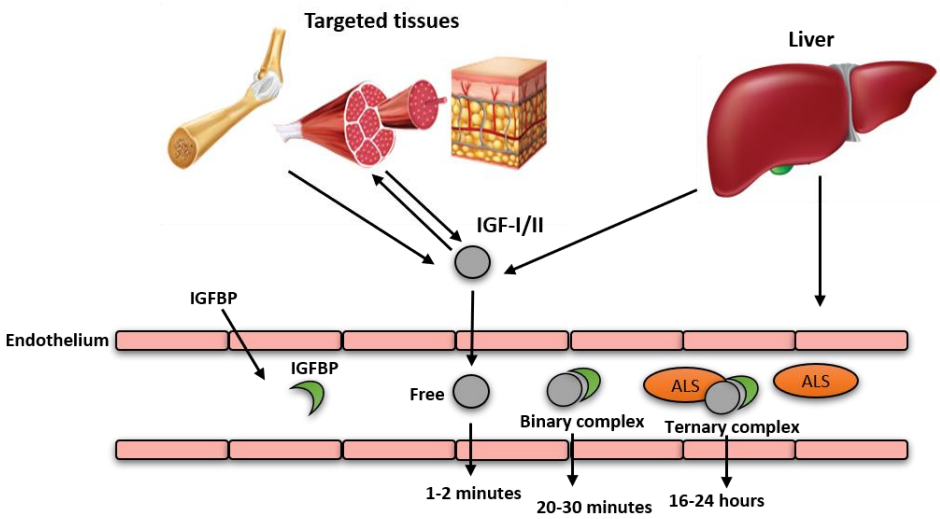


Figure 1-5 The insulin-like growth factor (IGF) system in the circulation

(adapted from (Baxter 2014))

IGFs are mainly secreted by the liver and can also be produced by other organs. They act in an autocrine or paracrine manner and in addition to their presence in the circulation, they are found in binary complexes with IGFBP-1–6. Binary complexes with IGFBP-3 and IGFBP-5 can form a ternary complex with the acid-labile subunits (ALS). The figure illustrates the circulating half-lives of free and bound IGF.

The binding between IGFs and IGFBPs leads to a more stable complex with lower dissociation rates. However, this binding can be locally regulated by proteolytic enzymes (IGFBP-proteases) including matrix metalloproteinases, metalloproteinase 28 (ADAM 28) and pregnancy associated plasma protein A (PAPPA-1) (Nakamura, Miyamoto et al. 2005, Boldt and Conover 2011), which can specifically increase cleavage of the IGF binding proteins and alter IGF bioavailability (Laursen, Overgaard et al. 2001).

1.4 IGF system ligands

1.4.1 IGF-II

1.4.1.1 Structure

IGF-II is a 7.5 kDa peptide mainly secreted by the liver, with 62% homology with IGF-I and consists of 67 amino acids. It is expressed from the 30 kb IGF-II gene located on chromosome 11p15.5, interestingly, next to the insulin gene.

Serum levels of IGF-II remain stable during adult life, with a concentration of 700 ng/ml and only show a minimal decline with ageing (Bennett, Wahner et al. 1984, Humbel 1990). In comparison with IGF-I, its levels are more abundant in the serum and remain unchanged during pregnancy as it acts as an important regulator of fetal growth and development, but its physiological role in adults is less well understood.

Free IGF-II circulates at low concentrations (picomolar) and its biological activity follows binding to the IGF-IR, IR-A isoform and IGF-I/IR hybrid receptors. It acts in an endocrine, autocrine and paracrine manner and IGF-IR activation is thought mediate most of the biological effects of IGF-II. IR-A binds IGF-II with a greater affinity than IGF-I. It is generally known that activation of the IR-A receptor by IGF-II is mitogenic, whereas insulin binding generates a metabolic response (Frasca, Pandini et al. 1999). IR-A is broadly distributed, and its physiological function is yet to be fully determined.

The lack of clear understanding of the physiological role of IGF-II in humans may be due to its overlapping effects with IGF-I and the lack of naturally occurring inherited conditions that result in a deficiency of IGF-II (Braun, Bitton-Worms et al. 2011).

1.4.1.2 IGF-II regulation

IGF-II is precisely regulated and at many levels, its gene is regulated by imprinting; it is only expressed by the paternal allele in most tissues, and its maternal allele is not expressed normally. The imprinting control region (ICR) regulates the expression of IGF-II and H19 genes in somatic cells, in the maternal cells ICR is hypomethylated and will bind to the nuclear protein CCCTC-binding factor (CTCF), which acts as an insulator protein. This binding occurs at four sites leading to the inhibition of IGF-II proximal promoter activity due to the isolation from its distal enhancer. In paternal cells however, the ICR is methylated leading to CTCF binding inhibition, this will promote IGF-II expression through its connection with the downstream enhancer (Szabó, Tang et al. 2004) as shown in Figure 1-6. This regulation of IGF-II methylation and expression is dysregulated in multiple pathological conditions including obesity and cancer (Chen, Ip et al. 2000, Cui, Onyango et al. 2002).

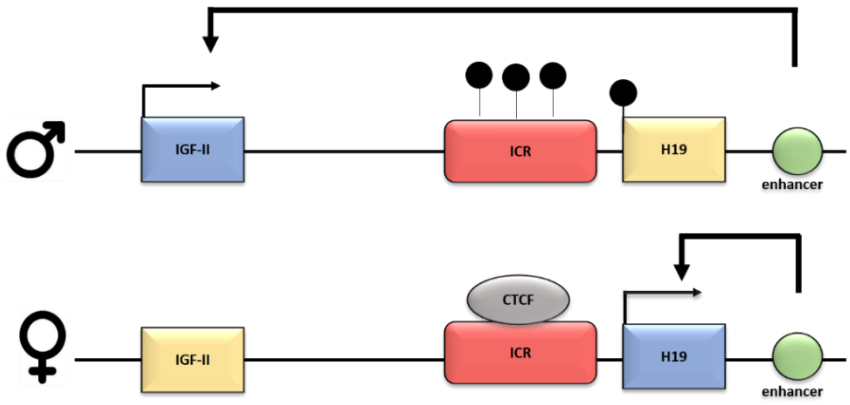


Figure 1-6 Model of IGF-II imprinted genetic expression (*adapted from (Kameswaran and Kaestner 2014))*

The H19-IGF-II locus on the maternal allele has unmethylated ICR, which allows the attachment of the CTCF suppressor protein, causing loss of connection between the IGF-II promoter and its downstream enhancer. In the paternal allele, the ICR is methylated and the connection is persistent between IGF-II and its enhancer.

In the serum, IGF-II is bound to IGFBPs to regulate its bioactivity. This binding affects the free levels of IGFs, for instance, a higher concentration of IGFBP-1 was related to greater suppression of the IGF-II free level (Juul 2003). At the tissue level, IGF-II actions depend on the tissue expression of its targeted receptors as it binds the IGF-IR, the IR and with higher affinity to the IR-A: IGF-II also has its own receptor (IGF-IIR/M6P) which acts as a clearance receptor to promote further regulation of IGF-II levels (Kornfeld 1992). Unlike IGF-I, IGF-II is weakly regulated by growth hormone, as GH only influences its levels indirectly by increasing the production of IGFBP-3 from the liver and ALS ternary complex formation. This explains why IGF-II levels are not influenced by puberty (Wolf, Kramer et al. 1994).

1.4.1.3 The role of IGF-II in growth

IGF-II has a well-established role as an essential embryonic growth promoter: knocking out IGF-II in mice leads to severe growth retardation in utero but knocking out the same gene after birth had no effect on normal growth. Therefore, IGF-II is fundamental for normal embryonic growth but not crucial for postnatal development in mice (DeChiara, Efstratiadis et al. 1990). The role of IGF-II in humans may be more extensive, as unlike mice, in which the level of IGF-II is negligible after weaning, the serum levels in humans remains relatively high and constant throughout adult life (Soares, Ishu et al. 1985). Multiple studies have shown that IGF-II has a role in intra-uterine growth in humans, as it is abundantly secreted by the placenta; IGF-II helps to increase the formation of mesoderm cells, improve nutrient transport, trophoblast invasion and proliferation, survival of cytotrophoblasts and promotes organ development. IGF-II promotes angiogenesis through its regulation of vascular endothelial growth factor (VEGF), which stimulates differentiation of endothelial

cells (Irwin, de las Fuentes et al. 1993, Schwartz, Hudgins et al. 1993). The role of IGF-II postnatally is less understood, however, a recent report suggested that IGF-II is also important in growth promotion in humans after birth (Begemann, Zirn et al. 2015). Other studies also suggest a role in postnatal bone development and cartilage growth (Uchimura, Hollander et al. 2017).

1.4.1.4 The role of IGF-II in metabolism

Physiologically IGF-II is reported to have insulin-like effects; its overall actions are anabolic and result in the lowering of blood glucose levels: IGF-II acts on the liver to reduce hepatic glucose output and increase glucose storage as glycogen. In muscles, IGF-II also helps to decrease blood glucose level by facilitating glucose uptake and oxidation, and stimulating the synthesis of both lipids and proteins (Rajpathak, Gunter et al. 2009). IGF-II serum levels are related to the nutritional status of the individual, as its levels are decreased with malnutrition (Wolf, Kramer et al. 1994) and increased with obesity, both total (Buchanan, Phillips et al. 2001) and free IGF-II (Frystyk, Skjaerbaek et al. 1999); this change in serum levels being reversible with weight loss (Belobrajdic, Frystyk et al. 2010). IGF-II was indicated as a prognostic marker to predict future weight gain in a study that followed both sexes for four years. The study showed that higher levels of IGF-II at baseline were protective against future weight gain (Sandhu, Gibson et al. 2003). In addition, higher levels of IGF-II and IGFBP-2 were associated with increased HDL cholesterol in diabetic patients (Narayanan, Fu et al. 2013).

In genetic studies, many associations between IGF-II polymorphisms and weight have been reported. A study of 1,474 men demonstrated that Apal AA is associated with lower body weight but higher serum IGF-II levels (O'Dell, Miller et al. 1997), but this was contradicted by another study indicating no difference in BMI or fat mass between

Apal AA and other IGF-II genotypes (Roth, Schrager et al. 2002). Furthermore, it has been suggested that high in utero expression of the paternal IGF-II allele may cause fat deposition postnatally in the offspring (Le Stunff, Fallin et al. 2001), and the level of IGF-II methylation at birth may contribute to the development of obesity and weight gain in early childhood (Perkins, Murphy et al. 2012) .

Adipocytes have the ability to secrete more IGF-II than IGF-I (Gude, Frystyk et al. 2012). The metabolic actions of IGF-II in terms of glucose uptake and inhibition of lipolysis are less potent than IGF-I and insulin as demonstrated in an *in vitro* culture study using the 3T3-L cell line. This was explained by the lesser affinity of IGF-II for the IGF-IR in comparison to IGF-I (Siddals, Westwood et al. 2002). In primary adipocytes where the IR is additionally expressed, IGF-II was more potent in activating the insulin receptor than IGF-I (Bäck, Brännmark et al. 2011). With this strong association with body weight, and the high expression of IGF-II in adipose tissue, it would be interesting to explore if IGF-II has regulatory actions on fat cells, especially on visceral adipocytes as they express a higher level of insulin receptors than subcutaneous adipocytes and these are predominantly the IR-A isoform (Lefebvre, Laville et al. 1998).

1.4.2 Insulin

Insulin plays a major role in regulating glucose uptake and metabolism. It is secreted from the pancreatic β-cells into the blood and acts as an anabolic hormone that promotes glucose uptake and storage mainly through its actions on the liver, muscles and adipose tissue.

Insulin maintains blood glucose levels within a narrow range by increasing glucose absorption from the blood to the liver and fat tissue by promoting the translocation of

the glucose transporter (GLUT4) from the cytoplasm to the cell surface. The anabolic effects of insulin include promoting glycogen formation, protein synthesis and lipogenesis. By contrast, insulin reduces lipolysis, glycogenolysis and protein breakdown and inhibits the production of glucose from the liver. In addition to its metabolic effects, insulin also promotes cell growth and differentiation (Alan and Kahn 2001).

Insulin is composed of two polypeptide chains, which consist of an A chain with 21 amino acids and the B chain has 30 amino acids; the chains are linked together by two disulphide bonds, and an extra disulphide bond is created within the A chain. The additional C-peptide chain is cleaved when insulin becomes biologically active (De Meyts 2004).

Insulin is essential for survival and any disturbances in insulin levels can lead to metabolic instability in the form of diabetes, with high fasting and postprandial glucose and elevated free fatty acids (Shoelson, Lee et al. 2006).

1.4.3 IGF-I

IGF-I shares 50 to 70% structural similarity to insulin. It is predominantly secreted by the liver into the circulation (Schwander, Hauri et al. 1983). IGF-I is also expressed in other tissues including adipocytes (Frystyk 2004). The primary regulator of IGF-I is growth hormone (GH) and it is important in cell proliferation, growth, apoptosis and migration, thereby affecting organism size and longevity (Holt, Simpson et al. 2003). The adult serum levels of IGF-I reach around 100–200 ng/ml. These levels vary with age, being half that of adult levels at birth and increasing to adult levels around puberty, however the highest level of IGF-I is found during puberty, with an almost three fold increase in concentration (Humbel 1990, Frystyk 2004). This increase

during puberty is accompanied with the pubertal-GH surge, after which levels decline. The pubertal surge is associated with an increase in IGFBP-3 levels, but with a maintained predominance in IGF-I secretion leading to an overall increase in free IGF-I (Juul, Dalgaard et al. 1995).

Knockdown of the IGF-I gene in mice caused deficient embryonic growth and consequent dwarfism and infertility in the offspring (Adams, Epa et al. 2000). In humans, loss of functional IGF-I causes pre- and postnatal growth retardation and occasionally, developmental retardation and partial or total deafness (Duyvenvoorde, Losekoot et al. 2007).

Although IGF-I is generally considered a key postnatal growth factor, as it stimulates the growth of several cell types, it also has other significant metabolic effects either by its direct effect on targeted tissues or indirectly through facilitating the actions of GH. IGF-I lowers blood glucose levels by directly promoting tissue glucose uptake in muscle and supressing glucose production from the liver. In addition, IGF-I helps to increase insulin sensitivity (Guler, Zapf et al. 1987) and has an antilipolytic effect on adipose tissue, promoting protein synthesis (LeRoith and Yakar 2007) .

The GH-IGF axis is a fundamental regulator of childhood growth, influenced by multiple factors, including nutritional and hormonal regulation. Obesity is associated with decreased levels of GH, and increased adiposity, predisposing more to central adiposity (Berryman, Glad et al. 2013). The levels of GH are inhibited by increased concentrations of IGF-I, insulin and free fatty acids. Obesity may alter the GH-IGF axis through increasing insulin in the portal veins in association with insulin resistance, the suppression of IGFBP-1, which may increase free levels of IGF-I (Lewitt, Hilding et al. 2008) leading to reduction in GH. Furthermore, in obesity there is an amplified IGF-I response to GH and an increase in GH receptor expression

leading to the absence of IGF-I suppression. However, total IGF-I levels showed inconsistent associations with obesity; levels were lowered, increased or showed no changed in obese individuals (Rasmussen, Hvidberg et al. 1995). Furthermore, the increase in FFAs associated with obesity can cause a reduction in GH levels. Interestingly, the reduction in FFAs by the use of the antilipolytic drug ‘ acipimox’ lead to a restoration of GH release (Cordido, Peino et al. 1996).

1.5. IGF binding proteins (IGFBPs)

IGF binding proteins (IGFBP) are major regulators of the bioavailability of IGFs as the IGFBP-IGF binding complexes help to transport IGFs in the circulation, facilitate their movement out of the vascular compartment, elongate the half-life of the hormones and inhibit them from binding to their corresponding receptors. There are six high affinity IGFBPs (1–6), which are structurally related. They bind IGFs with high affinity but do not bind to insulin (Wetterau, Moore et al. 1999). The affinity of IGFBPs is almost similar for IGF-I and IGF-II, however some binding proteins have preferable binding, like IGFBP-6 which is reported to have a higher affinity for IGF-II (Rechler 1993).

IGFBPs have various functions and different levels of expression, their main function is to regulate the action of IGFs, but they can also function independently of the IGFs, as regulators of cell growth, apoptosis and gene transcription (Lawrence, Oxvig et al. 1999, Wetterau, Moore et al. 1999). The most abundant IGFBP in serum is IGFBP-3 and its binding to IGFs is highly regulated by GH (Juul, Dalgaard et al. 1995). IGFBP-2, the second most abundant binding protein in serum, and IGFBP-4 have overall inhibitory actions, while the effects of IGFBP-1 varies, inhibiting the actions of IGFs

when phosphorylated and facilitating their effects when dephosphorylated (Jones and Clemmons 1995).

1.5.1 IGFBPs in adipogenesis

IGFBPs have an important role in adipogenesis and metabolism, both directly and indirectly through the regulation of IGFs. IGFBP-1 inhibited the effect of IGF-I on the differentiation of preadipocytes, and IGFBP-1 overexpression in mice helped to reduce diet-induced obesity (Rajkumar, Modric et al. 1999, Siddals, Westwood et al. 2002). However, when IGFBP-1 was overexpressed in mice, it led to the development of age-related glucose intolerance. IGFBP-1 levels change during the day, with low levels after meals, that correlates with its regulation by insulin (Crossey, Jones et al. 2000).

IGFBP-2 is secreted by white preadipocytes during the process of adipogenesis (Boney, Moats-Staats et al. 1994) and its serum levels decrease with obesity both in adults (Nam, Lee et al. 1997) and in children (Frystyk, Skjaerbaek et al. 1999, Ballerini, Ropelato et al. 2004). Studies have also shown that high IGFBP-2 expression can be protective against obesity and its related complications (Wheatcroft, Kearney et al. 2007). In addition, IGFBP-2 also has fat-depot specific differences as visceral adipocytes secrete more of this binding protein, and silencing IGFBP-2 promotes adipogenesis and lipogenesis in visceral but not subcutaneous white adipocytes (Yau, Russo et al. 2014).

Interestingly, in addition to their IGF-dependent effects, IGFBPs have also been reported to exert adipose tissue IGF-independent biological actions. IGFBPs were found to act independently by having their own cellular receptors and/or regulate nuclear interactions, and in adipose tissue, IGFBP-3 reduced 3T3-L1 preadipocyte differentiation in a non-IGF-I-dependent manner (Chan, Schedlich et al. 2009). This

was confirmed using an IGFBP-3 mutant (has less affinity to IGF-I) that showed similar results to the wild-type IGFBP-3 (Chan, Schedlich et al. 2009). IGFBP-3 binds directly to RXRα, causing regulation of PPARγ transcription by modulating RXRα PPARγ dimerisation. IGFBP-2 also has IGF independent nuclear binding interactions but the consequences of this have still not been defined (Miyako, Cobb et al. 2009). Table 1-2 summarises the major actions of IGFBPs in adipose tissue.

Table 1-2 Role of IGF binding proteins in adipose tissue regulation *(Baxter and Twigg 2009)*

IGFBP	Regulatory action	References
IGFBP-1	Inhibit IGF-I stimulated differentiation in 3T3-L1 preadipocytes Inhibits IGF-I stimulated clonal expansion in 3T3-L1 preadipocytes Impaired adipogenesis in IGFBP-1 transgenic mice	(Nueda, García-Ramírez et al. 2008) (Siddals, Westwood et al. 2002) (Rajkumar, Modric et al. 1999)
IGFBP-2	Moderate expression level in rat adipose tissue Inhibit IGF-1 induced differentiation in 3T3-L1 preadipocytes	(Gosteli-Peter, Winterhalter et al. 1994) (Wheatcroft, Kearney et al. 2007)
IGFBP-3	Inhibited preadipocyte differentiation in 3T3-L1 preadipocytes Reduced insulin stimulated glucose uptake in 3T3-L1 adipocytes and human visceral adipose tissue Stimulated preadipocytes differentiation in preadipocytes from children	(Chan, Schedlich et al. 2009) (Chan, Twigg et al. 2005) (Grohmann, Sabin et al. 2005)
IGFBP-4	Moderate expression in rat adipose tissue Higher expression in visceral adipose tissue in comparison to subcutaneous tissue in fetal baboon preadipocytes	(Gosteli-Peter, Winterhalter et al. 1994) (Tchoukalova, Nathanielsz et al. 2009)

IGFBP-5	Highly expressed in porcine adipose tissue Expression is reduced with differentiation	(Hausman, Poulos et al. 2006) (Gardan, Mourot et al. 2008)
IGFBP-6	No published data	

1.6 IGF receptors

1.6.1 The IGF-I (IGF-IR) and insulin receptor (IR)

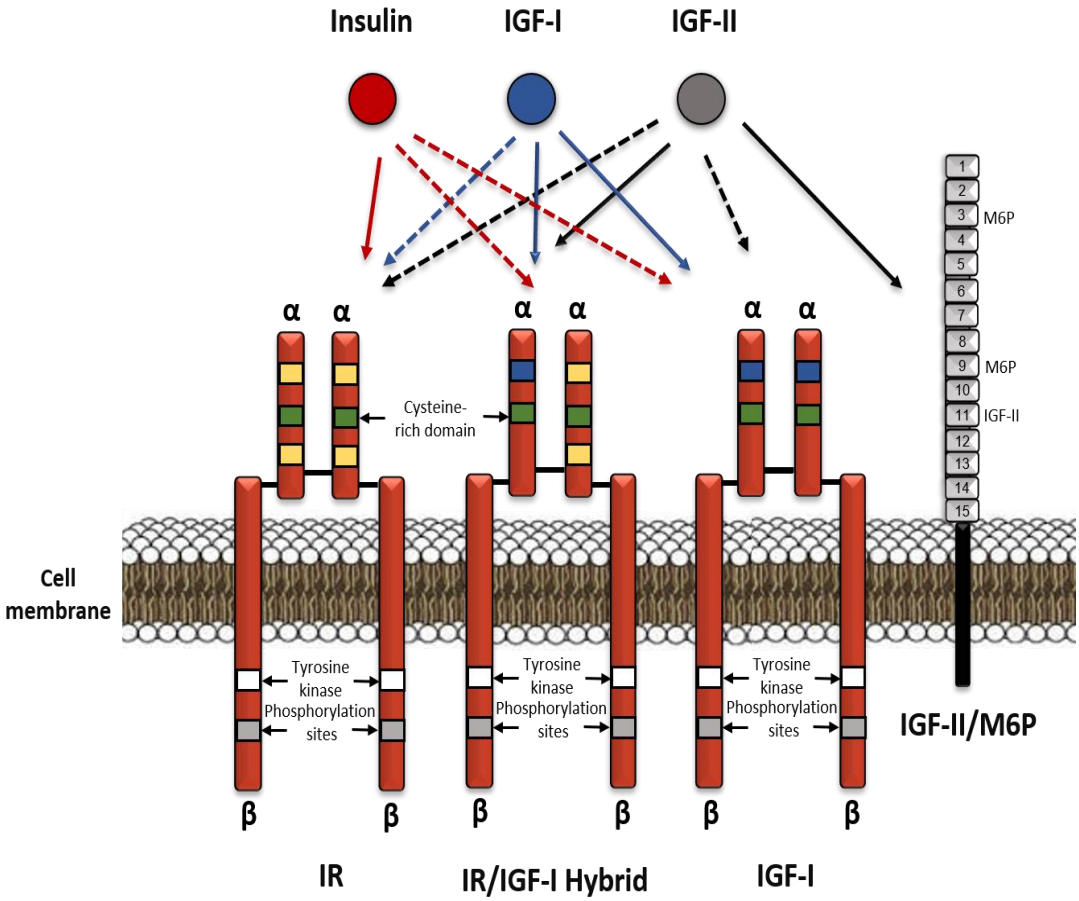


Figure 1-7 Structure of the IGF receptors *(adapted from (Hawkes and Kar 2004))*

The structure of insulin, IGF-I, insulin/IGF-I hybrid and IGF-II/M6P receptors. IGF-I and insulin receptor are highly similar in structure and both belong to the tyrosine kinase receptor family. This cell surface hetero-tetramer receptor consists of two α and two β subunits connected by disulphide bonds. The IGF-I/IR hybrid receptor is composed of IGF-I α β and insulin α β hemi-molecules. IGF-II/M6P receptor is a transmembrane receptor composed of an amino-terminal signal sequence, large extra-cytoplasmic domain, a single transmembrane region and a cytoplasmic tail.

1.6.1.1 Receptor structure

The IGF-I (IGF-IR) and IR receptors belong to the tyrosine kinase family of receptors and they share almost 84% structure similarity. These two cell membrane receptors are composed of a pair of α and β subunits connected by disulphide bonds (Siddle, Ursø et al. 2001). The α subunits are located extracellularly and contain the ligand binding sites, whereas the β subunits are transmembrane and have tyrosine kinase domains (Werner, Weinstein et al. 2008) as illustrated in Figure 1-7. What determines the physiological responses to specific- receptor activation is still being investigated but in general, the IGF-IR is thought to mediate mitogenic effects, with the IR mediating metabolic actions. Each receptor has higher affinity for their corresponding ligand but additionally, they also bind to the other ligand with lower affinity. IGF-II can bind to the IGF-IR (6-fold lower affinity than IGF-I) and to insulin receptors with different affinities depending upon the isoform of the insulin receptor present (Chisalita, Nitert et al. 2006, Werner, Weinstein et al. 2008). Studies also showed differences in intracellular receptor signalling, ligand dissociation rates and RTK domains that influence the signal outcome (Blakesley, Scrimgeour et al. 1996, Sasaoka, Ishiki et al. 1996, Dupont and LeRoith 2000, Kim and Accili 2002, Bouzakri, Zachrisson et al. 2006). The IR α subunit has two binding sites known as Site 1 and Site 2, when insulin binds, it activates the low affinity Site 1 on one α-subunit, then the high affinity Site 2 on the other α-subunit, binding to both sites are important for high affinity binding (Ward, Lawrence et al. 2007). For IGF-I only one α subunit binding site is required to bind the IGF-IR.

Knockout of the IR in mice had no effect on birth size but caused a rapid loss of metabolic function in terms of hyperglycaemia, hyperinsulinemia and pancreatic β cell failure leading to metabolic ketoacidosis and death. In humans, mutations of the IR

results in severe growth retardation at birth and postnatal developmental delay (Wertheimer, Lu et al. 1993, Accili, Drago et al. 1996, Nakae, Kido et al. 2001). In mice loss of the IGF-IR, severely affects growth and causes multiple abnormalities, leading to death within minutes, which clearly illustrates the importance of these two receptors (Nakae, Kido et al. 2001).

1.6.1.2 Receptor function

Activation of the IGF-IR or IR is associated with phosphorylation of two different pathways. Ligand binding leads to initial autophosphorylation, causing multiple substrate molecules like IRS-1, 2 and src homology 2/collagen alpha proteins (SHC) to bind. This results in phosphatidylinositol 3-kinase (PI3K)-AKT/protein kinase B (PKB) activation, which is mainly responsible for metabolic activities (Myers, Backer et al. 1992), and the mitogen-activated protein kinase (MAPK) pathway, which is involved in regulating cellular growth, proliferation and differentiation (Pouysségur, Volmat et al. 2002).

PI3K activation results in the conversion of PIP2 to PIP3, leading to the activation of AKT causing an increase in glucose uptake. Furthermore, activation of the pathway induces phosphorylation of 4EBP-1, leading to the release of eIF4E and promoting protein synthesis (Morita, Gravel et al. 2015). In addition, mTOR will lead to S6K phosphorylation, which also results in protein synthesis, and in addition activates anti-apoptotic transcription factors leading to lower cellular apoptosis (Magnuson, Ekim et al. 2012).

In contrast, the MAPK pathway is associated with SHC phosphorylation, and the formation of the GRB2 and SOS1 complex that activates Ras, MAP kinases, SRF and

ELK1 leading to mitogenic signalling and regulation of apoptosis (Siddle 2011). Figure 1-8 illustrates the signalling pathways of IGF-IR and IR.

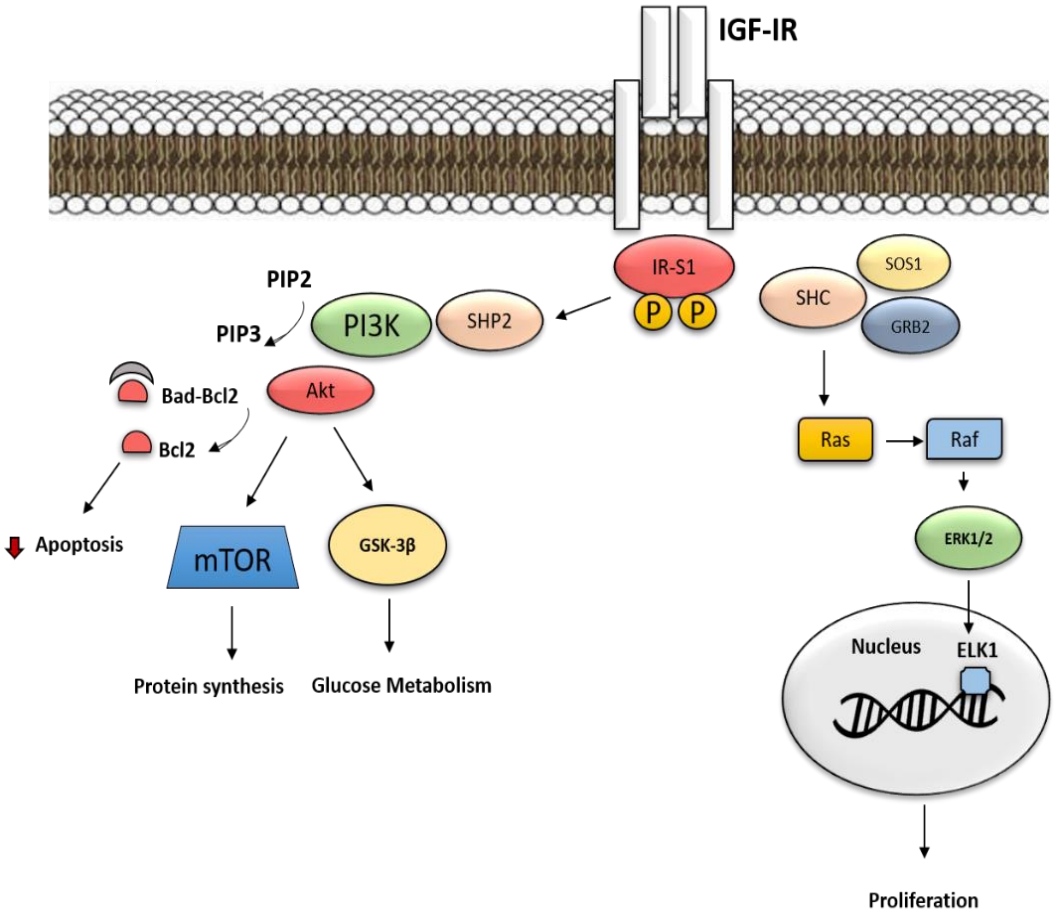


Figure 1-8 Insulin-like growth factor I (IGF-IR) and insulin (IR) receptor signalling pathways *(adapted from (Pollak, Schernhammer et al. 2004))*

Ligand attachment is associated with activation of two downstream pathways; PI3K pathway (left) which is involved in metabolic functions. MAPK pathway (right) which is involved in growth related functions.

1.6.2 Insulin-like growth factor-II/mannose 6-phosphate receptor (IGF-IIR/M6P)

1.6.2.1 Receptor structure

In contrast to the structure of the IGF-I and IRs, the insulin-like growth factor-II/mannose 6-phosphate (IGF-IIR/M6P) receptor is completely different. The IGF-II/M6P receptor is a type I transmembrane glycoprotein with no intrinsic tyrosine kinase activity. The receptor protein structure consists of three domains; the N-terminal extracellular region which is comprises 15 repeated segments of 147 amino acids, each segment has a cationic-dependent mannose-6-phosphate receptor, which resembles the M6P receptor in structure, but only the third and the ninth repeated segments have M6P binding sites. The IGF-II binding site-2 is located on the eleventh repeating segment (Figure 1-7). Interestingly, the IGF-II eleventh segment is close to the thirteenth segment, which has a fibronectin type II-like insert, that enhances IGF-II binding to its binding segment (Marron-Terada, Hancock et al. 2000). Furthermore, the extracellular region in the repeating segments have cysteine residues that facilitate the formation of disulphide bonds required for receptor folding (Hille, Waheed et al. 1990, Dahms and Hancock 2002).

The second receptor domain is the single membrane spanning region and the last region is the cytoplasmic tail which possesses phosphorylation sites for multiple kinases, for example protein kinase C (PKC) and casein kinase I, II (MacDonald, Coussens et al. 1988, Körner, Nürnberg et al. 1995). The receptor is also present in the cytoplasm and can be recycled to the cell surface by multiple factors like IGF-I and insulin (Hawkes and Kar 2004).

1.6.2.2 Receptor function

The IGF-II/M6P functions as an endocytosis facilitator for M6P-ligands to be activated or degraded in the lysosomes by the receptor's ability to carry the newly formed lysosomal enzymes to the trans-Golgi network (TGN) to be placed at targeted organelles or moved extracellularly.

The other well recognised function of IGF-II/M6P is to act as an IGF-II clearance receptor, which regulates the circulating levels of IGF-II (Rodríguez, Gaunt et al. 2004). IGF-IIR/M6P knockout in the mouse was associated with fetal over growth (Hébert 2006). Nevertheless, inhibition of the IGF-IIR/M6P was not associated with a decrease in the growth promoting actions of IGF-II in cardiac cells (Hawkes and Kar 2004), and the receptor was not involved in the mitogenic effects of IGF-I or IGF-II in fibroblasts (Furlanetto, Dicarlo et al. 1987). Yet, IGF-IR receptor suppression caused a reduction in IGF-I related signalling (Hébert 2006). The biological effects of IGF-II signalling through the IGF-II/M6P receptor is still unclear, mainly because of the absence of tyrosine kinase activity.

The IGF-IIR/M6P is involved in cell proliferation and cell differentiation as other ligands have the capacity to bind this receptor. For example, transforming growth factor-β (TGF-β), which is known as an inhibitor of cell growth and differentiation in many cells (Hawkes and Kar 2004, Hébert 2006), inhibits differentiation of preadipocytes to mature adipocytes (Sparks, Allen et al. 1992, Petruschke, Röhrig et al. 1994). Another IGF-IIR/M6P ligand is the cellular repressor of E1A-stimulated gene (CREG), which regulates differentiation and also acts as a repressor of cellular proliferation (Han, Guo et al. 2008). The CREG inhibitory effects were reported in smooth muscle cell growth *in vitro* (Han, Liu et al. 2005) and in NIH3T3 fibroblasts (Han, Guo et al. 2008).

1.7 Molecular basis of the role of IGF in adipogenesis

Adipogenesis is a multi-levelled cascade involving multiple transcription factors. This process involves lineage commitment of mesenchymal stem cells, then preadipocyte expansion, followed by preadipocyte differentiation. Transcription factors C/EBP and PPARγ are important in initiating the signalling cascade to promote adipocyte differentiation: PPARγ initiates transcription by dimerising to RXR alpha (Rosen and MacDougald 2006). The process is further described in section 1.2.2.

IGF-I is secreted by the liver, and also secreted locally by the adipose tissue. It acts as an important regulator of preadipocyte growth and has a stimulating effect on adipogenesis *in vitro* (Smith, Wise et al. 1988, Christoffersen, Tornqvist et al. 1998). In comparison to proliferating preadipocytes, differentiating preadipocytes have suppressed IGF-I induced MAPK activation via the MEK inhibitor, leading to adipogenesis stimulation in 3T3-L1cell lines (Boney, Gruppuso et al. 2000). IGF-I is also involved in the clonal expansion of 3T3-L1 (Siddals, Westwood et al. 2002). IRS-1/2 signalling is important in adipogenesis as IRS-1/2 deficient fibroblasts have dysregulated PI3K signalling and this is associated with lower adipogenic expression of the differentiation markers PPARγ and C/EBP (Miki, Yamauchi et al. 2001). The importance of IGF-I in adipose tissue growth was also observed when a reduction in IGF-IR abundance was associated with multiple organ growth deficits, particularly for adipose tissue causing a reduction in preadipocyte proliferation (Holzenberger, Hamard et al. 2001). In addition, an AKT-knockout mouse model exhibited failed adipogenesis and dysregulation of the transcription factor PPAR (Peng, Xu et al. 2003). Reports have also indicated that IGF-I can oppose the effect of Pref-1 which acts as a powerful suppressor of adipogenesis and is highly expressed in preadipocytes (Zhang, Nøhr et al. 2003). Taken together, this indicates that IGF-I is an important

regulator of adipogenesis, mainly mediated through activation of the IRS-1/PI3K/AKT pathway. Dysregulation of this pathway is associated with pathogenesis and obesity (Maccario, Tassone et al. 2001, Gleeson, Lissett et al. 2005). The mechanism of IGF-I action in preadipocyte growth is illustrated in Figure 1-9.

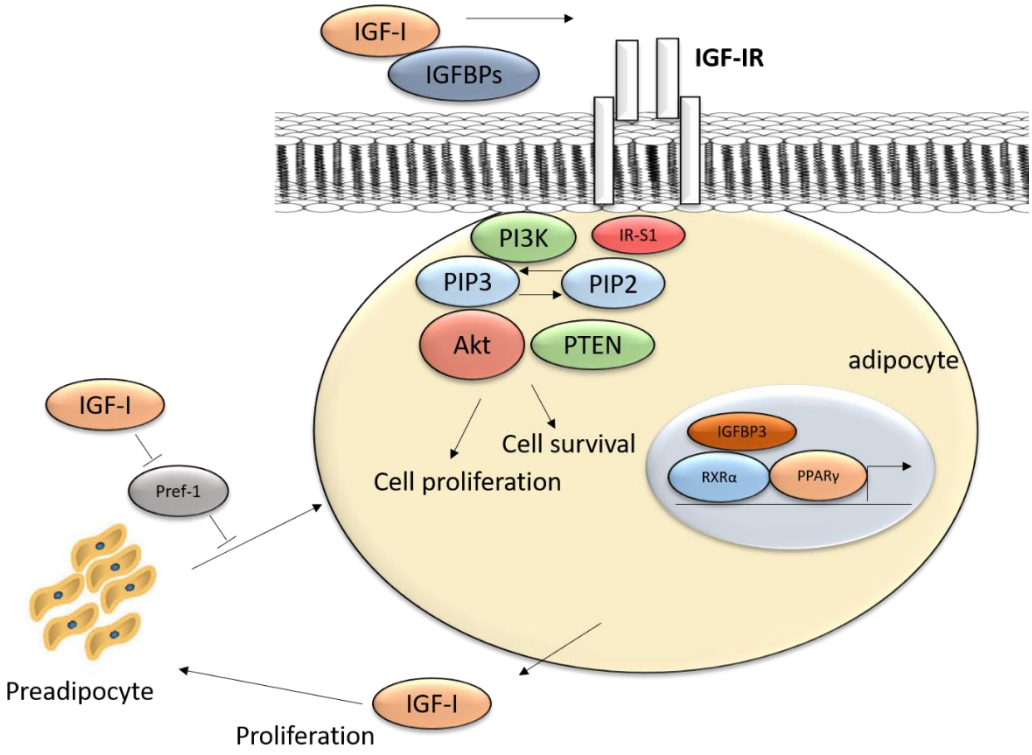


Figure 1-9 Illustration of the role of the IGF system in preadipocyte growth *(adapted from (Kawai and Rosen 2010))*

IGFBPs regulates IGF-I availability to its cognate receptor, IGF-I activates PI3K pathway in adipogenesis. In addition, IGFBPs have IGF-independent actions; IGFBP-3 binds RXRα and regulates PPARγ transcriptional levels in adipocytes. IGF-I can also be locally secreted, acting in an autocrine/paracrine manner.

1.8 Insulin receptor isoforms

1.8.1 Structure and function

The insulin receptor was initially reported to have two sequences that differed by 12 amino acids and more recently it was determined that this 12 amino acid difference was due to the alternative splicing of exon 11 leading to the formation of two IR isoforms, IR-B (11+) that included the 12 amino acid residue (residues 717-728) at the carboxyl end of IR α-subunit and IR-A (11-) that lacked the 12 amino acid residue, that enabled these isoforms to act differently in many functional and physiological aspects (Seino, Seino et al. 1989). Figure 1-10 illustrates the structural differences in the IR isoforms.

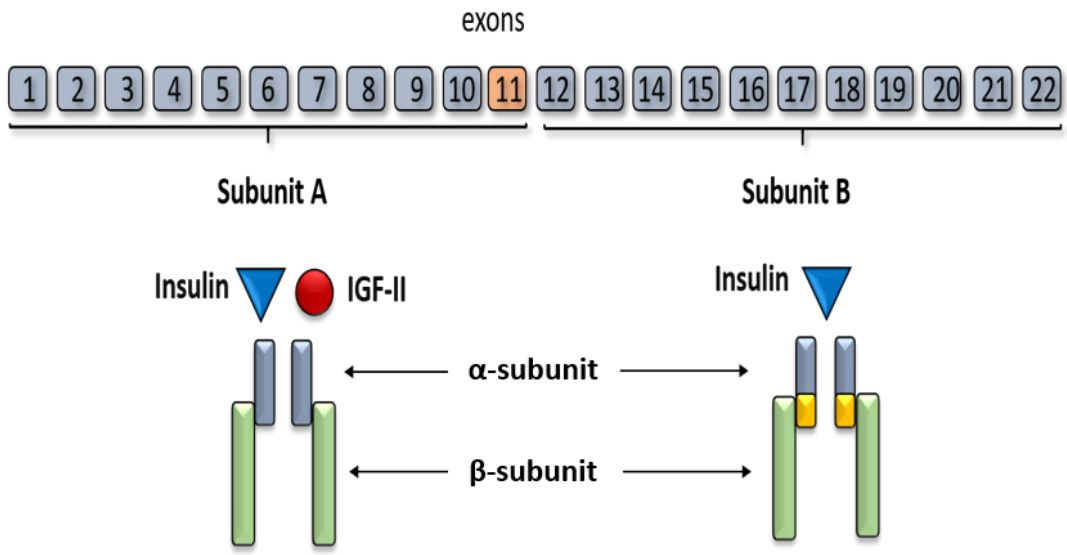


Figure 1-10 Structure of insulin receptor alternative splicing *(adapted from (Westermeier, Sáez et al. 2016))*

The insulin receptor gene (INSR) consists of 22 exons and is found on chromosome 19. The exclusion or inclusion of exon 11 in the insulin receptor mRNA, in the insulin receptor-α subunit, leads to the formation of two insulin receptor isoforms IR-A and IR-B.

The two isoforms vary in their tissue distribution. IR-A is generally considered a fetal isoform and is highly expressed in fetal tissues like liver, kidney and muscles. IR-B is highly expressed in adult tissues, mainly in liver, and is linked to tissue differentiation (Mosthaf, Grako et al. 1990). IR-A is also expressed in adult tissues with IR-B in tissues like fat and muscles (Belfiore, Frasca et al. 2009).

The alternative splicing also affects the receptor affinity for hormones; IR-A has higher affinity for insulin (1.7 fold) and IGF-II (10 fold) in comparison to IR-B, which has no affinity to IGF-II. In addition, IR-A is reported to have a higher internalisation rate in response to insulin, but IR-B has more efficient insulin signalling transmission (Vogt, Carrascosa et al. 1991, Kellerer, Lammers et al. 1992). Studies also indicate some differences in the signalling pathways used by these two isoforms. With pancreatic β cells, insulin promotes insulin production and β-glucokinase transcription through activation of the class I PI3-K pathway via IR-A. However, IR-B signalling occurs through the class II PI3-K pathway (Leibiger, Leibiger et al. 2001). IGF-II can induce different cellular outcomes depending upon the receptor that mediates its actions: in haematopoietic cells activation of IR-A by IGF-II protects the cells from apoptosis, whereas IGF-II activation of IR-B was more powerful in inducing cell differentiation (Sciacca, Prisco et al. 2003). Table 1-3 summaries the major differences in IR isoform structure and function.

Table 1-3 Summary of the major differences between insulin receptor isoform A and B

IR-A	IR-B	References
Predominant in fetal tissues and adult rat: pancreas, brain placenta and spleen	Predominant in human adult tissues and in tissue targets of the metabolic effects of insulin	(Moller, Yokota et al. 1989) (Goldstein & Dudley, 1990) (Sugimoto et al., 2000), (Serrano, Villar et al. 2005), (Frasca, Pandini et al., 2008)
High affinity to insulin and IGF-II with stronger affinity to IGF-II than insulin	No affinity to IGF-II	(Vogt et al., 1991), (Kellerer et al., 1995)
Higher insulin internalisation rate	More insulin signal efficiency	
Mitogenic, IR signalling from endosomes	Metabolic, IR signalling from plasma membrane	(Giudice, Leskow et al., 2011)
Stimulates MAPK pathway and ERK1/2 phosphorylation	Has higher AKT phosphorylation after insulin stimulus	
Linked to cancer and insulin resistance		(Frasca et al., 1999b) (Sciacca et al., 1999) (Savkar et al., 2001)

1.8.2 Insulin receptor isoforms in growth and metabolism

Insulin receptor isoform expression varies developmentally and in a tissue-related manner. The change in receptor expression is important in the regulation of growth and tissue function. Preadipocytes express the IR and the IGF-IR, with an increasing expression of IR with differentiation. Interestingly, a study in BAT, where either IR or IGF-IR were knocked out, showed that each receptor has a distinct regulatory role in promoting adipogenesis and the regulation of associated signalling molecules (Bäck and Arnqvist 2009, Bäck, Brännmark et al. 2011). Furthermore, in a fat-specific IR and IGF-IR knock out (FIGIRKO) mouse model, both insulin and IGF-I were needed for WAT and BAT growth. The unique receptor distribution change with differentiation indicates that the IGF-IR is less important in adipocytes when they became more insulin dependent (Boucher, Mori et al. 2012). In support of this, knockout of the IR in mature adipocytes causes a reduction in adipose tissue and associated insulin resistance, whereas IGF-IR deletion had minimal effects on fat mass and on glucose uptake (Yakar, Kim et al. 2005), indicating the importance of insulin signalling in mature adipocytes (Sakaguchi, Fujisaka et al. 2017).

Insulin receptor isoforms also change with differentiation, in mice brown adipocytes, the IR-B ratio increased with differentiation (Serrano, Villar et al. 2005); this pattern of isoform shift was also observed in hepatic cells HepG2 *in vitro* (Kosaki and Webster 1993) and *in vivo*, where IR-B expression was increased by 35% from fetal to adult liver (Giddings and Carnaghi 1992). This alternative splicing phenomena and the shift in insulin receptor isoform expression is also observed in other tissues, like osteoblasts in which precursor cells express high levels of IR-A but express more IR-B on differentiation to mature osteoblasts (Avnet, Perut et al. 2012). Similar changes in

isoform expression have been observed in the mammary gland (Neville, Webb et al. 2013) and in alveolar cells (Rowzee, Ludwig et al. 2009).

This pattern of IR isoform shift could also indicate a distinctive functional role between these isoforms, the important role of these isoforms in the physiological regulation of tissue growth and differentiation. This is most likely through the ability to mediate different effects of insulin and IGF. Although some reports examined the IR isoform change with differentiation in adipose tissue, little is mentioned about fat-depot differences in expression. This has mainly been reported in mouse models indicating a difference in isoform distribution (Serrano, Villar et al. 2005, Vienberg, Bouman et al. 2011), or similar amounts of IR-A and IR-B between adipose cells of different fat-depots (Vienberg, Bouman et al. 2011). Although humans have more distinct fat-depot differences than mice and fat distribution is more related to metabolic health risks, there are less reports indicating the receptor isoform expression and as far as we known no reports indicated fat depots differences in children. IR-A was expressed more in human fetal fibroblasts and IR-B was increased in adults adipocytes (Blanquart, Achi et al. 2008), others indicated higher IR-A levels in human fibroblasts and adipocytes (Moller, Yokota et al. 1989) .

In metabolism, the higher expression and phosphorylation of IR-B in hepatic HepG2 cells in comparison to IR-A indicates that IR-B might be more involved in metabolic regulation (Kosaki, Pillay et al. 1995). Liver specific insulin receptor knock out (LIRKO) mouse models in which IR-B predominates, show dysregulation of glucose and lipid metabolism through effects on glucose homeostasis, gluconeogenesis and lipid production. However, in specific IR-B but not IR-A knockout, IR-A was shown to compensate for IR-B actions in the liver (Diaz-Castroverde, Baos et al. 2016). The use of isoform insulin specific analogues showed that IR-B was more related to

glycogen regulation in liver and muscles, and lipogenesis in adipose tissue, whereas IR-A was more associated with glycogen synthesis in muscles (Vienberg, Bouman et al. 2011). Although overall studies indicate a more potent metabolic role of IR-B, the role of insulin receptor isoforms in human metabolism is still not clear.

Interestingly, dysregulation of IR isoforms was noted in multiple diseases in relation to growth and metabolism. The altered insulin receptor ratio favouring increased expression of IR-A has been found in many cancers for example, breast, lung and colon (Frasca, Pandini et al. 1999, Sciacca, Costantino et al. 1999). Furthermore, changes in receptor isoform expression are associated with metabolic pathologies like diabetes, with IR-A expressed to a higher degree than IR-B in liver (Huang, Bodkin et al. 1996) and muscle tissue (Huang, Bodkin et al. 1994) of diabetic monkeys. However, human reports are inconsistent showing an increased expression of IR-B/IR-A in fat and muscle from obese and diabetic patients (Sesti, D'Alfonso et al. 1995), higher IR-A/IR-B in muscle of diabetic patients (Norgren, Arner et al. 1994) and similar expression of the two isoforms in skeletal muscle (Benecke, Flier et al. 1992). In addition, myotonic dystrophy patients show a lower level of IR-B in muscle tissue and this was associated with impaired insulin responses (Savkur, Philips et al. 2001). These inconsistences in the reports of IR isoform expression in diabetes may be attributed to the complexity of the disease, hyperglycaemia, age, stage of the disease and genetic variation (Escribano, Beneit et al. 2017).

1. 9 General Hypothesis

With a limited understanding of the postnatal role of IGF-II and the lack of data on children, this study aimed to investigate the physiological role of IGF-II by conducting a series of experiments using primary cultures of matched pairs of subcutaneous and visceral adipocytes from children to test the hypothesis that IGF-II is an important regulator of adipocyte physiology with specific effects on visceral adipocytes. It was hypothesised that the differential distribution of IR isoforms, particularly IR-A between visceral and subcutaneous adipocytes will allow IGF-II to have a profound effect of visceral cells leading to a fat-depot differences on adipose cell expansion and metabolism.

1.10 Study aims and objectives

- We aim to study fat-depot receptor distribution in children, by characterising the differences in IGF receptor (IGF-IR, IR, IR-A and IR-B) expression between subcutaneous and visceral preadipocytes, and differentiated adipocytes.
- Investigate the effect of IGF-II on preadipocyte growth, by the assessment of proliferation and differentiation in a fat-depot specific manner.
- Determine the differences in IGF-II regulation in subcutaneous and visceral cells by examining the roles of the IGF-IIR/M6P and IGFBP-3.
- Evaluate the role of early IGF-II serum levels as a biomarker of body fat distribution in children during puberty by using data from ALSPAC lifecourse cohort.

Chapter 2 : Material and methods

2.1 Adipose tissue biopsy collection

2.1.1 Ethical issues

The study was approved by the NRES Committee South West – Exeter (REC reference: 14/SW/0109 on 3rd July 2014. An invitation to the study and a written information sheet were sent by post (along with the surgery admission letter) to all potential subjects. Parents/legal guardians were approached by Dr.Maiadah Alfares for a verbal explanation of the study on the morning of admission for surgery. Children aged over 5 years were also given a simple information sheet explaining the basics of the research. They were then left, for at least 60 minutes, to consider whether or not they wanted to participate. All of those recruited signed three written consent forms: one for the hospital records, one for the patient to keep, and one for the study files. Approval of the study, information sheets and the consent form are supplemented in Appendix (9.1-9.4).

2.1.2 Subject recruitment

Participants for this study were recruited from the paediatric urology surgical ward who were attending the Royal Hospital for Children (Bristol) for non-malignant and non-septic operations that require intra-abdominal access. All the recruited children were between 0 and 8 years old and operations such as elective pyeloplasties, nephrectomies and hemi-nephrectomies were best for providing paired subcutaneous and visceral adipose tissue samples. It was anticipated that most children recruited using this method would have a relatively ‘normal’ weight for age and sex. Our aim was to establish a cryopreserved bank of paired subcutaneous and visceral fat cultures from 20 subjects.

Exclusion criteria: Children undergoing surgery for any emergency and those in whom surgery did not necessitate intra-abdominal access as part of the procedure. Families who might not adequately understand verbal explanations or written information given in English were also excluded.

Data collection: Prior to surgery the child had his or her height and weight measured as per routine and the following details were recorded: date of birth, date of operation, birth weight and gestation, gender, and ethnicity.

2.1.3 Sample collection

Fat tissue samples were collected by the Consultant Paediatric Surgeons in the Bristol Royal Hospital from children at the beginning of the operation. Two pea-sized pieces of subcutaneous and visceral fat biopsies (0.2–0.5g) were collected and transferred immediately to the laboratory in a solution composed of phosphate-buffered saline (PBS) and 20mg/ml bovine serum albumin (BSA).

2.2 Tissue and cell culture techniques

2.2.1 Equipment

General sterile cell culture plastic was purchased from Greiner Bio-One (Gloucestershire, UK), serological pipettes were acquired from Corning Incorporated (Amsterdam, Netherlands), Pasteur pipettes were bought from Fisher Scientific (Loughborough, UK), syringes were from Terumo (Leuven, Belgium), and 0.2µM filters were from Appleton Woods (Birmingham, UK). Cryogenic vials were obtained from NUNC (Roskilde, Denmark) and the haemocytometer was from Hirschmann (8100104). The incubators were from SANYO (MCO-18AIC), hoods were from

BIOMAT (Class II, Medical Air Technology, Manchester, UK), and the centrifuge was from MSE (Centaur2).

2.2.2 Tissue culture reagents

All reagents were obtained from Sigma-Aldrich (Gillingham, UK) unless stated otherwise.

- **Penicillin/streptomycin** – Penicillin ($1x10^6$ IU/vial) and streptomycin sulphate (1g/vial).They were stored at -20°C in 5ml aliquots (Lonza, Berkshire, UK).
- **L-glutamine –** 14.6g of L-glutamine in 500ml sterile distilled water, filter-sterilised and stored at -20°C in 5ml aliquots (Lonza, Berkshire, UK).
- **Phosphate-buffered saline (PBS)** – PBS tablets (Oxoid Ltd., Basingstoke, UK) were used with one tablet/100ml of dH_2O. The prepared solution was sterilised by autoclaving at 121°C for 15 minutes and then stored at room temperature.
- **Trypsin EDTA solution (TE)** – 10x TE (Lonza, BE 02-007E, Berkshire, UK) was diluted with sterile ddH2O to make 1x. Then the solution was aliquoted into 5ml tubes and stored at -20°C.
- **Dimethylsulfoxide (DMSO)** – Sigma D8779.
- **Trypan blue** – Lonza, 17-942E, 0.4% (diluted to a working solution of 0.165% with PBS).
- **Hank's balanced salt solution (HBSS)** – Gibco 14060-040 (Paisley, UK).
- **Type II collagenase** – Sigma C6885.

- All plasticware used in the culture and differentiation of the human adipocytes was coated with 0.2% gelatine solution: 1g gelatine (Sigma G9391, Gillingham, UK) in 500ml distilled water and autoclaved.
- Tissue culture medium used for the culture of primary human preadipocytes and a mouse 3T3-L1 fibroblast cell line are listed in Table 3.

Table 2-1 Media components used for culture of primary human preadipocytes and 3T3-L1 fibroblast cell line (Green and Meuth 1974, Grohmann, Sabin et al. 2005).

Cell type	Media components
3T3-L1 growth media	Dulbecco's modified Eagle's medium (DMEM) supplied with either normal (5mM, 1g/L) or high (25mM, 4.5g/L) glucose obtained from BioWhittaker (Verviers, Belgium), supplemented with 4mM L-glutamine, 10% NCS (Gibco, Paisley, UK), 5000U/ml penicillin, and 5000µg/ml streptomycin.
3T3-L1 differentiation media	Dulbecco's modified Eagle's medium (DMEM), 4.5g/l glucose or normal glucose (1g/L), 25mM HEPES, supplemented with 4mM L-glutamine, 10% US certified FBS, 5000U/ml penicillin, and 5000 µg /ml streptomycin (Lonza, Berkshire, UK).
	Days 0–3: basic differentiation media + 0.25µM dexamethasone (Sigma D8893), 166nM insulin (Novo Nordisk, West

	Sussex, UK), and 0.5nM IBMX (Sigma I5879).
	Days 3–5: basic differentiation media + 166nM insulin.
	Days 5–7: basic differentiation media only.
3T3-L1 preadipocyte serum-free media (SFM)	DMEM supplemented with 4mM L-glutamine, 5000U/ml penicillin, and 5000µg/ml streptomycin.
Human preadipocyte growth media	DMEM/Ham's F12 with 15mM Hepes and 15mM NaHCO3 (Gibco 31330-038), supplemented with 20% HiFBS, 5000U/ml penicillin, 5000µg/ml streptomycin, and 1% L-glutamine (Lonza, Berkshire, UK).
Basic preadipocyte differentiation media	DMEM high glucose (4.5g/L) or normal glucose (1g/L) supplemented with 33µM biotin (Sigma B4639), 10µg/ml apotransferrin (Sigma T2036), 1µM dexamethasone (Sigma D8893), 0.2nM 3,3,5.-triiodothyronine (Sigma T6397), 17µM pantothenate (Sigma P5155), 5000U/ml penicillin, and 5000µg/ml streptomycin, 1% L-glutamine, and 1% fungizone (Fisher Scientific 10658154, Paisley, UK).
	Days 0–3: basic differentiation media + 100nM insulin + 25uM IBMX + 10uM Rosiglitazone (Sigma R2408).
	Days 3–10: basic differentiation media + 100nM insulin.
	Days 10–14: basic differentiation media only.

Human preadipocyte serum-free media (SFM)	DMEM/Ham's F12 with 15mM Hepes and 15mM NaHCO3, supplemented with 5000U/ml penicillin, 5000µg/ml streptomycin and 1% L-glutamine.

2.2.3 General cell culture techniques

2.2.3.1 Cell passaging

- Cells were washed with PBS before detaching the cells with trypsin (1ml/T75 flask, 5ml/T175 flask).
- Cells were incubated with trypsin for 5 minutes at 37°C.
- Trypsin was deactivated by adding fresh growth media (5ml/T75 flask, 10ml/T175), and the cell suspension was centrifuged at a relative centrifugal force (rcf) of 80g for 5 minutes to pellet the cells.
- The supernatant was aspirated, and the cells were resuspended in 5ml of fresh growth media; this process represented an increase in the passage number of the cells by 1. Cells were then either counted for plating or seeded into other flasks for further growth.

2.2.3.2 Cell counting (trypan blue dye exclusion assay):

After trypsinisation, 50µl of cell suspension were mixed with an equal volume of 0.4% trypan blue solution (Lonza, 17-942E) that was loaded into both chambers of a clean haemocytometer with an adherent coverslip (Hirschmann, 8100104), as shown in **Figure 2-1**. Viable bright cells in the centre square were counted under a light microscope (dead cells will appear as dark blue). Viable cells in the centre square of

the top and bottom grids were counted at a x10 magnification using an EMM single hand counter (Jencons, 670-032), which were added together and then multiplied by $x10^4$ to give the total number of cells per ml of cell suspension.

Figure 2-1 Haemocytometer slide for cell counting.

A haemocytometer with a glass coverslip showing the top and bottom counting grids (right) and a haemocytometer diagram indicating the area of counting under the microscope (left).

2.2.3.3 Cryopreservation and resurrection

2.2.3.3.1 Cryopreservation

Cells were grown to 80–90% confluency in either T75 or T175 flasks, trypsinised, counted, and then centrifuged at 80g for 3 minutes. After removing the supernatant, cells were resuspended in the appropriate cell-specific media containing 10% DMSO at a concentration of either $5x10^5$, $1x10^6$ or $2x10^6$ cells/ml, depending on the cell number. 1ml of cell mixture was put into each cryovial and appropriately labelled. The cryovials were then placed in a Mr. Frosty freezing container (Thermo Scientific) and stored at -80°C for 24 hours before being transferred to the liquid nitrogen storage tanks (Taylor-Wharton, LS48000).

2.2.3.3.2 Resurrection (thawing frozen cells)

Cells were removed from liquid nitrogen and rapidly thawed to ensure maximum cell viability by swirling the vial in a 37°C water bath for 1 minute. Once thawed, 1ml of growth media was added dropwise to the vial and then the cell solution was transferred into a universal tube containing 4ml of growth media. This mixture was placed into a T175 or T75 containing 25ml or 10ml of growth media. Following 24 hours of incubation at 37°C, to allow the cells to attach, the media was replaced with fresh growth media to remove any traces of DMSO and dead cells.

2.2.4 Culture and differentiation of 3T3-L1 fibroblast mouse cell line

3T3-L1 cells are a continuous sub-strain of 3T3 (Swiss albino) cells developed by clonal isolation and they convert from fibroblast-like cells (preadipocytes) to adipocytes. They were obtained from ATCC® CL-173™ and were grown to 70% confluency in a T75 flask. For experimentation, cells were plated into a 24-well plate with a seeding density of $2x10^4$ or into six-well plates at a seeding density of $8x10^4$ per well (according to the manufacturer's instructions) and induced to differentiate over 7 days using a differentiation media described in Table 3.

2.2.5 Isolation and culture of primary subcutaneous and visceral preadipocytes

The technique of preadipocyte isolation, culture and differentiation was developed within our laboratory and fully characterised by Dr. M. Grohmann (Grohmann, Sabin et al. 2005). The isolation of subcutaneous and visceral preadipose cells (Figure 2-2) was performed using an adapted method of Hauner et al. (Hauner, Entenmann et al. 1989) as follows:

- The adipose tissue was washed three times in 10ml of Hank's balanced salt solution (HBSS) and then was cut into $1mm^3$ pieces.
- Tissue was digested with 10ml of 1mg/ml type II collagenase in HBSS for 60 minutes at 37°C in a shaking water bath (150 cycles/min).
- Adipocytes were separated from the stromal vascular cells through centrifugation at 100g for 3 minutes.
- The pellet of sedimented preadipocytes (typically <1000 cells) was resuspended in preadipocyte growth media and seeded onto a T75 coated with 0.2% gelatine and maintained at 37°C in a humidified atmosphere of 5% CO2.
- Media was changed every 72 hours until confluency was obtained (7–14 days) and then cells were passaged into three T175 flasks. Upon confluency, 50% of the cells were frozen and the remainder were used for the experiment.

For differentiation, subcutaneous and visceral preadipocytes were seeded in six-well plates and after 16 hours they were induced to differentiate for 14 days using the preadipocyte differentiation medium described in Table 3. The experimentation

seeding densities were $0.017x10^6$ for the 24-well plate and $0.2x10^6$ for the six-well plate. To maintain consistency, experiments were performed using cell passages 3–5.

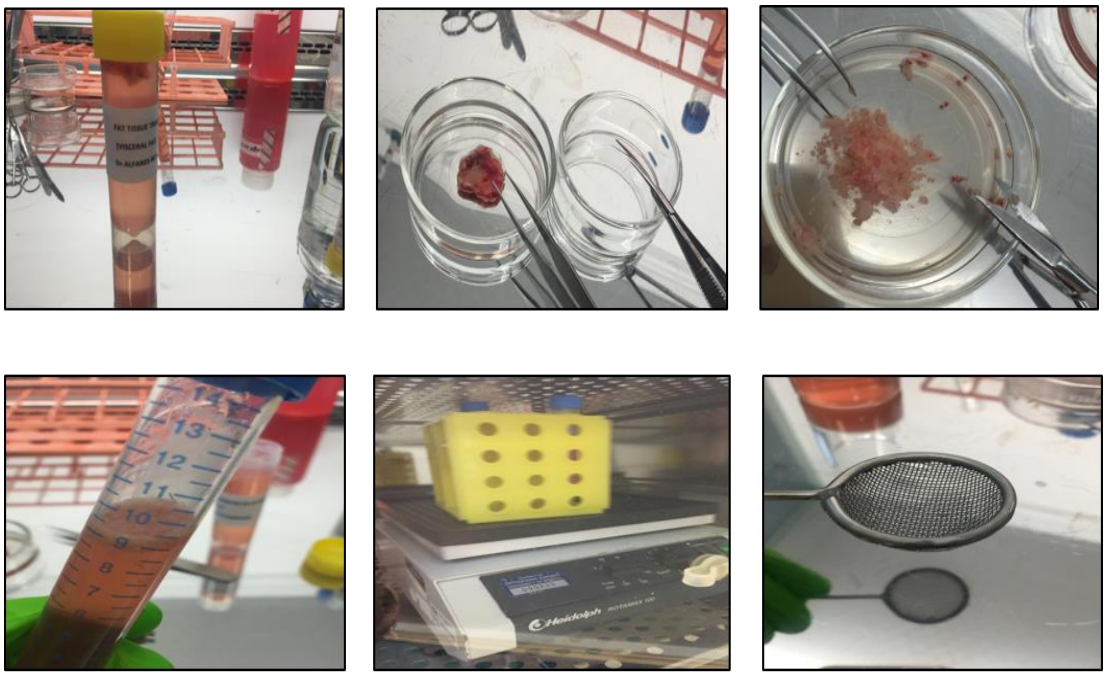


Figure 2-2 Preparation of subcutaneous and visceral fat samples obtained from children.

Collected visceral and subcutaneous fat samples were washed, dissected into small pieces and digested in type II collagenase at 37°C. The stromal vascular cells were then filtered, centrifuged, and the sedimented preadipocytes were resuspended in growth media and cultured.

2.3 Oil Red O triglyceride staining

2.3.1 Principle

Oil-soluble dyes help to stain lipids by being more soluble in natural fats than in the solvent in which they are dissolved. The oil-soluble dyes are strongly coloured and not soluble in water.

2.3.2 Procedure

Oil Red O (ORO) was used to evaluate the level of preadipocyte differentiation, and ORO stain stock was made up by dissolving 0.25g Oil Red O stain powder (Sigma O0625) in 50ml of isopropanol which had been stored for up to 6 weeks in the dark. A working solution of ORO was made by adding 10ml of ORO stock solution to 6.67ml of distilled water; this solution was then mixed and left for 10 minutes before being syringe-filtered to remove any large residual particles. The working solution had a shelf life of 2 hours and was made up fresh before use. Fully differentiated cells were washed twice in PBS before being fixed in 10% formalin for 10 minutes. Cells were then stained with the ORO working solution for 10 minutes. The stain was then removed, and the cells were washed a further two times with 60% isopropanol for 10 seconds per wash to eliminate any excess stain. The cells were then washed with distilled water and viewed using light microscopy.

Following the image capture, the level of staining was quantified by leaching the stain with 100% isopropanol (1ml/well) followed by spectrophotometry (FLUOstar Optima, BMG LABTECH) at 490nm.

2.4 Western immunoblotting

2.4.1 Protein extraction from cells

Following trypsin cell detachment from the culture flasks, cells were centrifuged, and the desired cell pellets were obtained as described in Section 2.2.3.1. Lysis buffer was used to release the proteins of interest. It was made with the following reagents: 10mM Tris HCL, 50mM sodium chloride, 5mM EDTA, 15mM sodium pyrophosphate, 50mM sodium fluoride, 1% Triton X-100, and 100µM sodium orthovanadate, and was adjusted to pH 7.6 and stored at 4°C. 10µl/ml of the protease inhibitor cocktail (Sigma P8340) and 10µl/ml of the phosphatase inhibitor cocktail (Sigma P5726) were added fresh to the lysis buffer before it was used. The lysis buffer mixture was made up to an appropriate volume and added to the cell pellet in an Eppendorf, resuspended and incubated on ice for 5–10 minutes. The cells were centrifuged at 80g for 5 minutes at 4°C and the cell extract was moved to a pre-cooled microcentrifuge tube and stored at -20°C for later use.

2.4.2 Protein concentration estimation

2.4.2.1 Principle

The total protein concentration within cell lysates was measured using a bicinchoninic acid assay (BCA) colorimetric reaction. The importance of this is in standardising the concentration of proteins between samples when loading to gels for western blot analysis. The principle of this assay is that protein peptide bonds cause a reduction in Cu^{2+} to a Cu^{1+} cuprous ion. Then the two bicinchoninic acid molecules chelate with the Cu^{1+} ion in an alkaline solution, leading to the formation

of a purple-coloured complex that can absorb light at a 540nm wavelength. The principle of the BCA assay is illustrated in Figure 2-3.

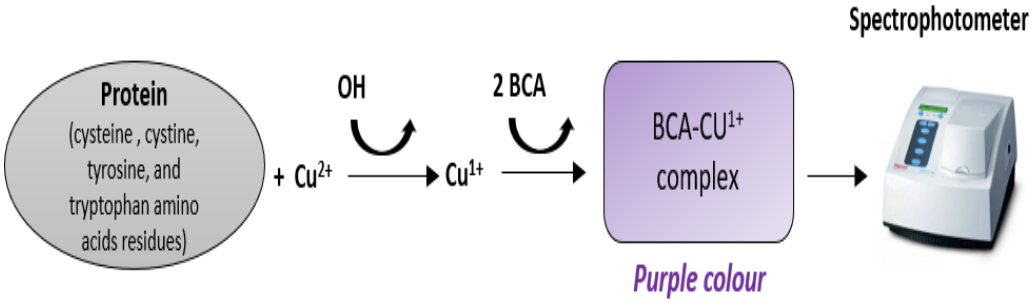


Figure 2-3 Principle of bicinchoninic acid assay (BCA). *Adapted from (Smith, Krohn et al. 1985).*

The proteins will induce a reduction of Cu^{2+} to a Cu^{1+} ion, and then the BCA will chelate with the Cu^{1+} in an alkaline environment, leading to the production of a purple-coloured complex. Absorbance will be measured by a spectrometer.

2.4.2.2 Equipment

- Plate reader (FLUOstar Optima, BMG LABTECH).

2.4.2.3 Reagents

- Bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL) containing:

1) **BCA Reagent A:** Sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide.

2) **BCA Reagent B:** 4% cupric sulphate.

- Albumin standard ampules, 2mg/ml, containing bovine serum albumin (BSA) (Fisher Scientific #BP9701-100, UK) at 2.0mg/ml in 0.9% saline and 0.05% sodium azide.

2.4.2.4 Procedure

Table 2-2 Protein standard concentration range.

Vial	Standard concentration	BSA (µl)	Lysis buffer
A	2000µg/ml	300µl of stock	0
B	1500µg/ml	375µl of stock	125
C	1000µg/ml	325µl of stock	325
D	750µg/ml	175µl of vial B	175
E	500µg/ml	325µl of vial C	325
F	250µg/ml	325µl of vial E	325
G	125µg/ml	325µl of vial F	325
H	25µg/ml	100µl of vial G	400

Protein standards were prepared by diluting a stock of 2mg/ml BSA solution with the same lysis buffer used to lyse the cells; the concentration range (0.025–2.0mg/ml) preparation is listed in Table 2-2. These were stored at -20°C until required. Before protein estimation, lysates were centrifuged at 14,000g for 5 minutes. 5µl of each standard was loaded into a 96-well plate in duplication, followed by a blank (5µl lysis buffer) in duplication and 5µl of each lysate in duplication. The plate layout is represented in Figure 2-4. BCA Protein Assay Reagents A and B were then mixed at a ratio of 50:1 and 200µl was added into each well. The samples were then incubated at room temperature for 30 minutes prior to spectrophotometric determination of colour change at 540nm using the MARS software package (BMG LABTECH). Protein concentrations were determined from the generated standard curve and the mean of the two values was used.

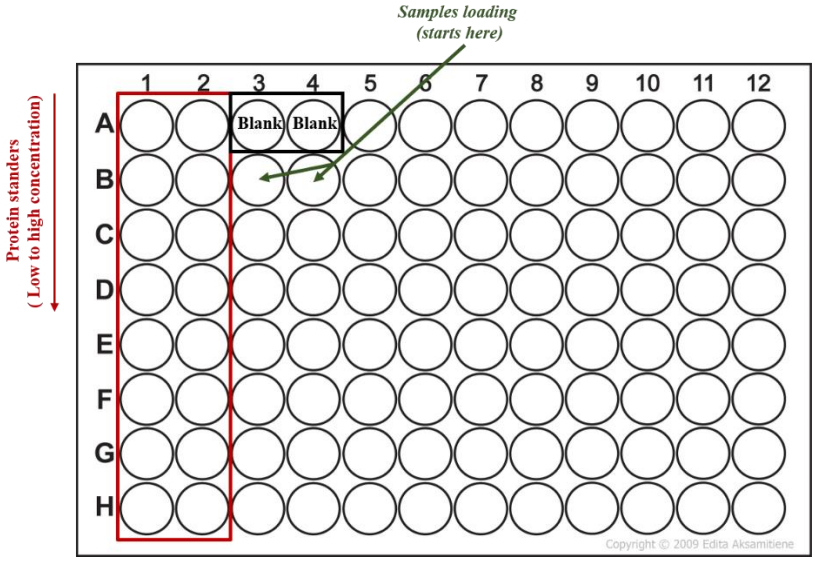


Figure 2-4 Layout of the 96-well plate used for protein concentration estimation assay.

Protein standards were loaded from low to high concentrations, followed by a blank (lysis buffer) and the unknown protein samples. All were added in duplicates.

2.4.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

2.4.3.1 Principle

SDS-PAGE is mainly used to separate complex protein mixtures according to size. Sodium dodecyl sulphate (SDS) is a detergent that solubilises hydrophobic molecules, and also carries a negative charge; thus, any cells that are incubated with SDS will be liberated of proteins because the membranes will be dissolved. The solubilised proteins are covered with negative charges and will migrate towards the positive electrode when placed in an electric field.

Specific percentages of acrylamide in the gel can be used depending on the size of the protein and the degrees of separation needed. Correct polymerisation of the gel is essential, as it forms a cross-linked polymer matrix which is used to support and

separate the molecules. The polymerisation catalysts incorporated into the gel are ammonium persulphate (APS) and N,N,N',N tetramethylethylenediamine (TEMED), the latter of which triggers the formation of persulphate free radicals from the APS which initiate the acrylamide polymerisation.

2.4.3.2 Equipment

- The Mini-PROTEAN Tetra cell system (including glass plates, combs, clamps, gel tank with lid and buffer dam, casting base and frames, and sample loading guides) (Bio-Rad 165-8000).
- Power pack (Pharmacia, LK-BS5 500/400).

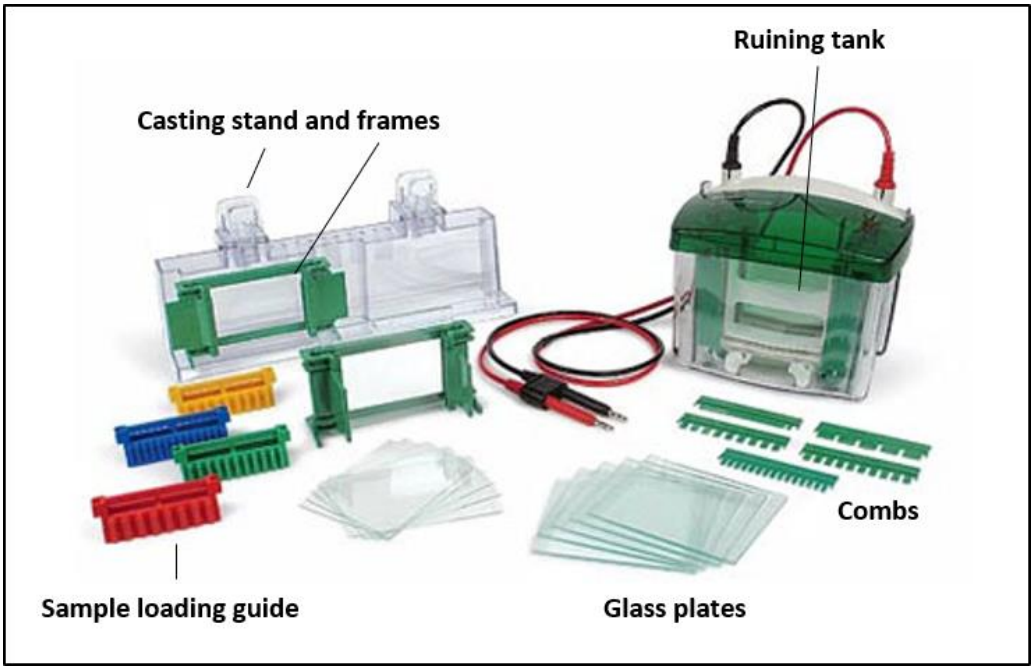


Figure 2-5 Western blot SDS-PAGE gel running equipment. *Picture obtained from www.bio-rad.com.*

The running system helps to separate proteins according to the molecular size. The electrical current will run through the buffer and this will force the negatively charged protein molecules to run towards the positive anode.

2.4.3.3 Reagents

Table 2-3 Western blotting reagents.

Reagent	Description
40% Acrylamide Gel Solution Bis-Acrylamide (Geneflow #A2-0075, UK)	Ratio of 37.5:1 and stored at 4°C.
(1.5M pH 8.8) Tris Base (Thermo Fisher Scientific #BP152-1, UK)	18.16g of Tris base was added into 70ml dH2O. Then the pH was adjusted to 8.9 before the solution was made up to 100ml and stored at 4°C.
(0.5M pH 6.8) Tris Base (Thermo Fisher Chemicals #1185-53-1, UK)	17.33g of Tris base was added into 70ml dH2O. Then pH was adjusted to 6.8 before the solution was made up to 100ml and stored at 4°C.
10% SDS (Fisher Scientific #BP166-500, UK)	50g was aliquoted into 400ml of gentle warmed dH2O. The pH was adjusted to 7.2 before the solution was made up to 500ml and stored at room temperature.
10% APS (Sigma-Aldrich #A6761, UK)	1g was added into 10ml of ddH2O and stored at 4°C. The solution needed to be used within 3–4 months.
TEMED (Fisher Scientific #T/P190/04, UK)	Stored at room temperature.
Butanol (Fisher Scientific, UK)	Made up at a 1:1 ratio with dH2O.
10x Tris-buffered saline TWEEN 20 [TBS-T]	12.1g Tris base, 40g NaCl (Fisher Scientific #S/3120/65) and 5ml TWEEN 20 (Sigma-Aldrich #P2287, UK) dissolved in 500ml dH_2O. The solution was calibrated with HCl and NaOH for a pH value of 7.6.
1x TBS-T	100ml 10x TBST diluted in 900ml dH_2O.
3% BSA (Fisher Scientific #BP9701-100, UK)	1.5g BSA dissolved in 1x TBS-T.
5% BSA	2.5g BSA dissolved in 1x TBS-T.

5% milk (powder, non-fat dry milk)	2.5g milk (Sainsbury's) dissolved in 1x TBST.
2x Laemmli sample buffer	4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, and 0.125M Tris HCl; pH 6.8.

2.4.3.4 Procedure

The resolving and stacking gels were made according to Table 2-4. The resolving gel portions are poured between glass plates 1.5mm apart and allowed to polymerise under a layer of butanol, which ensures a smooth interface whilst speeding up the polymerisation process. Once the gel has set, the butanol overlay is poured off and the gel is washed with dH2O before pouring the stacking gel and inserting a 10-well comb. After the stacking gel has polymerised, the combs are removed and the gels are transferred to the running apparatus. The chambers and tank are filled with running buffer prior to loading with the samples.

Table 2-4 SDS-PAGE gel preparation.

	8% resolving gel (>200kDa)	**10% resolving gel** (50–200kDa)	**12% resolving gel** (<80kDa)	**5% stacking gel**
Distilled water (dH_2O)	10.6ml	9.6ml	8.6ml	6ml
40% acrylamide	4ml	5ml	6ml	1.25ml
Tris buffer	5ml (1.5M pH 8.8)	5ml (1.5M pH 8.8)	5ml (1.5M pH 8.8)	2.5ml (0.5M pH 6.8)
10% SDS	200µl	200µl	200µl	100µl
10% APS	200µl	200µl	200µl	100µl
TEMED	20µl	20µl	20µl	15µl

- ***10x running buffer:*** was made using Tris base 30.3g (BP152-1 Fisher Scientific, UK), glycine 144g (BP381-5 5kg Fisher Scientific), SDS 10g, and dH2O, which was made up to 1 litre and then 1x running buffer was prepared by taking 100ml of 10x running buffer and adding it into 900ml of dH2O.

2.4.3.5 Sample preparation

Samples were spun at 12,000g for 10 minutes at 4°C to allow any fat to solidify and be removed. Correct volumes according to protein concentration were aliquoted into Eppendorfs with an equal volume of 2x Laemmli sample buffer (Sigma Life Sciences, S3401-1VL) containing anionic detergent (SDS), glycerol to increase the sample density and bromophenol blue dye to enable visualisation. Samples were heated to 100°C using an AccuBlock™ Digital Dry Bath (Labnet International) and a heating block for 5 minutes to denature the proteins before loading onto the gel.

2.4.3.6 Electrophoretic separation

The gel was run by limited volts (125–200V) according to the number of gels in the tank and for 45–90 minutes based on the separation required and protein size.

2.4.3.7 Transfer of protein to membrane

2.4.3.7.1 Equipment

- Mini Trans-Blot electrophoretic cell (Bio-Rad 170-3930).
- Supported nitrocellulose membrane (Amersham, RPN119B).
- Thick paper (Bio-Rad 1703967).
- Power pack (Pharmacia, LK-BPS 500/400).

Figure 2-6 Mini Trans-Blot electrophoretic cell compartments. *Picture obtained from www.bio-rad.com.*

The transfer system is essential in promoting the migration of the proteins from the SDS-PAGE gel to the nitrocellulose membrane, which is facilitated by the electrical current run which moves the proteins towards the positive electrode. The cooling unit is needed to help prevent overheating.

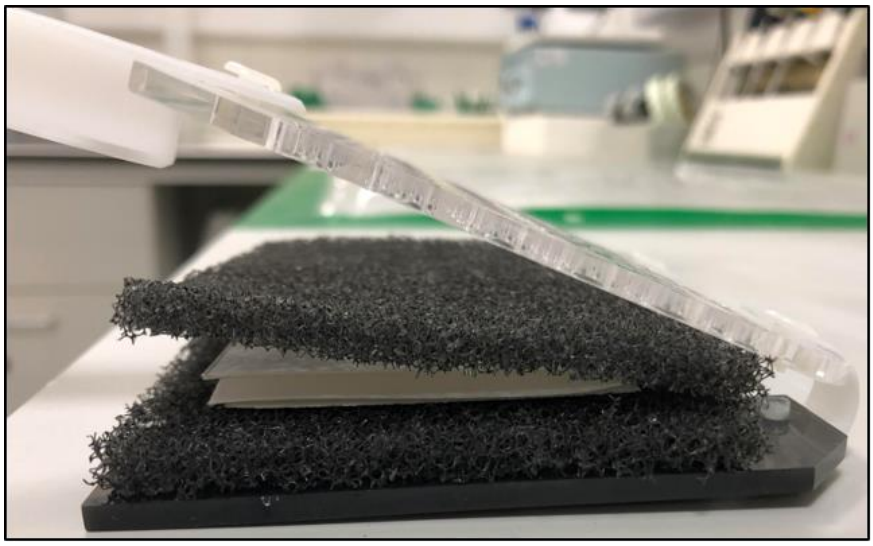
10x transfer buffer: Tris base 30.3g and glycine 144g were dissolved in dH2O to make up to 1 litre. Then 1x transfer buffer was prepared by taking 100ml of 10x

transfer buffer and adding 200ml of methanol (Fisher Scientific, M/3950/17, UK) and 700ml of dH2O.

2.4.3.7.2 Procedure

- Gels were removed from the running tank and soaked in 1x transfer buffer along with the nitrocellulose membrane, sponges and the thick paper for 10 minutes.
- A transfer sandwich was made with the following layout:

On the black side of the cassette: sponge – thick paper – gel – membrane – thick paper – sponge (as illustrated in Figure 2-6).



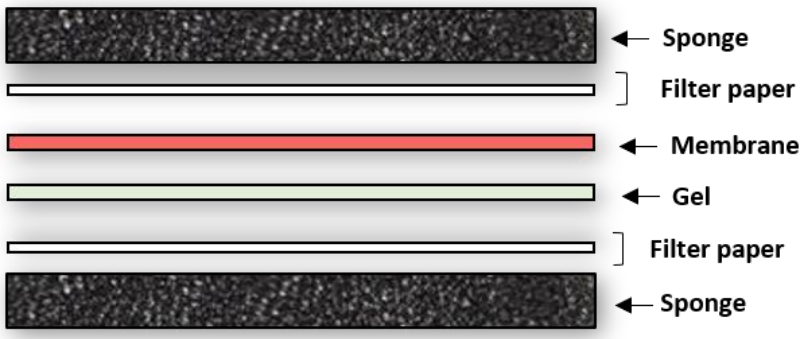


Figure 2-7 Layout of the western blotting transfer sandwich.

The sandwich is arranged by the demonstrated order, which allowed proteins to transfer from the SDS-PAGE gel to the supported nitrocellulose membrane.

- The sandwich was placed in the transfer tank, filled with 1x transfer buffer and was run by limited volts (90V) for 90 minutes.

2.4.3.8 Blocking

Blocking to prevent non-specific binding is achieved by incubating the membrane in a dilute solution of protein (typically 5% or 3% BSA or 5% non-fat dried milk in Tris-buffered saline TWEEN 20 [TBS-T]) for 1 hour at room temperature with gentle agitation. The protein from the solution then attaches to the membrane at all sites where the target proteins have not attached, so when the antibody is added the only places for it to attach to are the binding sites of the target protein. This helps to minimise background to ensure clearer results.

2.4.3.9 Primary antibody

After blocking, the membrane was probed with a primary antibody that was diluted in TBS-T containing 5% BSA, 3% BSA or 5% milk (according to the manufacturer's specification) and incubated overnight at 4°C. The properties of the antibodies used are listed in Table 2-5.

Table 2-5 Western blot antibody source, dilution and properties.

Antibody	Source	Block	Primary antibody dilution	Secondary antibody dilution	Protein size (kDa)
IGF-IR β	Cell Signaling Technology (9750)	5% milk	1:1000	αR 1:5000 (Sigma-Aldrich, A0545)	95
IR- β	Santa Cruz Biotechnology (C-19:sc-711)	5% milk	1:1000	αR 1:2000	97
PPAR- γ	Santa Cruz Biotechnology (E-8; sc-7273)	5% BSA	1:1000	αM 1:2000 (Sigma-Aldrich, A4416)	53–57
Adiponectin	Abcam (ab22554)	5% BSA	1:500	αR 1:2000	30
GLUT-4	Abcam (ab654)	5% milk	1:1000	αG 1:2000 (Sigma-Aldrich, A5420)	55
GLUT-1	Abcam (ab652)	5% milk	1:1000	αG 1:1000	55
FASN	BD Biosciences (610963)	3% BSA	1:5000	αM 1:2000	265
IGFIIR/M6P	Cell Signaling Technology (D3V8C)	5% milk	1:1000	αR 1:2000	275
IGFBP-3	Santa Cruz (sc-6003)	5% milk	1:1000	αG 1:2000	42–44
β-Actin	Sigma (A5441-100)	5% milk	1:10,000	αM 1:5000	40
GAPDH	Merk Millipore (MAB374)	5% milk	1:5000	αM 1:5000	35

Abbreviations: αM: anti-mouse antibody, αR: anti-rabbit antibody, αG: anti-goat antibody.

2.4.3.10 Secondary antibody

After washing in TBS-T for three 5-minute periods, the membrane was incubated with the corresponding secondary antibody (Table 2-5), which is a diluted horseradish peroxidase-conjugated (HRP) directed against the species in which the primary antibody was raised, for 60 minutes at room temperature. Then the washing steps were carried out again.

2.4.3.11 ECL detection

Following antibody incubation, the blots were washed three times for 5 minutes each at room temperature with TBST before the bands were visualised by SuperSignal West-Dura (Pierce, Rockford, IL) or Femto (Pierce, Rockford, IL) and incubated for 5 minutes with constant pipetting over the membrane and were visualised using the ChemiDoc XRS+ system and Image Lab software (170-8265, Bio-Rad, CA). Western blots were quantified using Image J 1.46r software (National Institutes of Health, Bethesda, MD) after scanning.

2.5 Tritiated thymidine incorporation assay (TTI)

2.5.1 Principle

There are multiple techniques with which to assess the level of cell proliferation and one of the most reliable and sensitive is that of directly measuring DNA synthesis. For that reason, we used a tritiated thymidine incorporation assay (TTI) to examine the rates of proliferation in primary human preadipocytes. In this assay a radioactive nucleoside, ^{3}H-thymidine, is combined into new strands of chromosomal DNA during mitotic cell division. A scintillation beta counter is used to measure the radioactivity in DNA recovered from the cells in order to determine the extent of cell division that has occurred in response to a test agent (Kaplan, Bott et al. 1992).

2.5.2 Equipment

- β-Scintillation Counter (Tri-Carb 2900TR, PerkinElmer Precisely, USA).
- Scintillation vials (Pony Vials, PerkinElmer, USA).
- Plate Rocker Platform (Bellco Biotech, NJ, USA).

2.5.3 Reagents

Table 2-6 Tritiated thymidine incorporation assay reagents.

Reagent	Description
[^{3}H]-thymidine (GE, Healthcare)	0.004µCi/ml stock (3µl of ^{3}H-thymidine in 750µl SFM).
Trichloroacetic acid solution (TCA) (Merck Ltd, Middlesex, UK)	5% TCA; 5g of TCA was added into 100ml ddH_2O and stored at 4°C.

1M sodium hydroxide solution (NaOH, S/4880/60, Fisher Scientific Ltd., Leicestershire, UK)	20g of NaOH was dissolved into 500ml ddH_2O and stored at room temperature.
Scintillation fluid	Ultima Gold, 6013329, Packard Bioscience Ltd.

2.5.4 Protocol

Cells were seeded onto 24-well plates in growth media and allowed to attach for 24 hours. Complete media was replaced with SFM for 24 hours. On the following day, cells were dosed with (7.81,15.62,31.25,62.5,125,250,500 ng/ml) of recombinant human IGF-I, IGF-II peptide (GroPep, Adelaide, Australia) or insulin (Novo Nordisk, West Sussex, UK). After 48 hours **(**as required), 0.004µCi of [^{3}H]-thymidine (25µl per well) was added for the last 4 hours at 37^{o}C, 5% CO_2. Media was removed and cells were incubated with 500µl 5% trichloroacetic acid (TCA) at 4^{o}C for 10 minutes. The TCA was removed and the cells were lysed in 500µl of 1M sodium hydroxide for 1 hour at room temperature on a plate rocker. The incorporation of [^{3}H]-thymidine into DNA was determined by scintillation counting; the sample (300µl) was mixed with 2ml of scintillation fluid and counted on a β-Scintillation Counter using QuantaSmartTM software. Data were recorded as disintegration per minute (DPM).

2.6 Quantitative polymerase chain reaction (qPCR)

2.6.1 Principle

The technique is dependent on primer-based enzymatic amplification of a targeted DNA sequence. The importance of this technique is in allowing a small DNA sample to be amplified into millions of copies complementary to the template strand. The steps of DNA generation are illustrated in Figure 2-8.

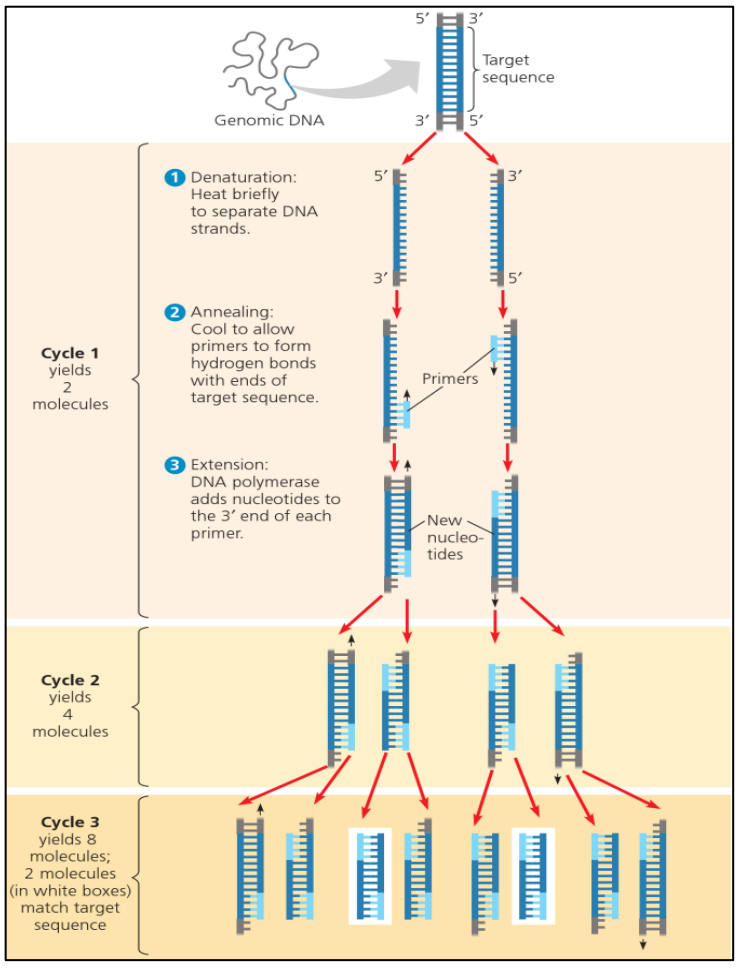


Figure 2-8 Quantitative polymerase chain reaction principle, indicating the processes of DNA amplification. *Picture adapted from www.laboratoryinfo.com.*

The DNA amplification is started by heat denaturing DNA to single strands, followed by primers annealing to their complementary sequence in the template DNA. Then, elongation will occur by DNA polymerase, which adds bases to the 3’ end of each primer, allowing

elongation in the 5’ to 3’ direction. One cycle will produce two separated pieces of double-stranded DNA, which will be further amplified in the following cycle.

2.6.2 RNA extraction

Cultured cells were washed with PBS twice and then 1ml of Trizol (Invitrogen, Auckland, New Zealand) per six-well plate was added whilst pipetting up and down repeatedly. The homogenised lysates were moved to an RNase-free 1.7ml Eppendorf tube and incubated for 5–7 minutes at room temperature to ensure complete dissociation of nucleoprotein complexes. Then, 0.2ml of chloroform (Fisher Scientific, 10293850) was added to each Eppendorf. Samples were then shaken vigorously for 15 seconds and incubated for 10 minutes at room temperature. Following centrifugation with a relative centrifugal force of 12,000g (PrismR, Labnet International) for 15 minutes at 4^{o}C, the upper colourless aqueous layer containing the RNA was transferred to new Eppendorfs (DNA and protein were left behind in the interphase and organic phases respectively). Then, 0.5ml of isopropanol (Fisher Scientific, 10284200) was added into the Eppendorf and incubated at room temperature for 10 minutes. The samples were centrifuged at 12,000g for 10 minutes at 4^{o}C. The supernatant was removed carefully and the RNA pellet was washed with 70% ethanol (Fisher Scientific, 10437341) and air-dried shortly before dissolving in around 10–12µl of RNase-free water (Thermo Scientific, SH30538.01).

2.6.3 RNA quantification

RNA was quantified using a NanoPhotometer (Implen, Munich, Germany). The LabelGuard Microliter Cell was inserted into the cell holder and the nucleic acid measurement option was selected in the Nanovolume application; the system was calibrated first through the use of 1µl RNase-free water applied into the centre of the

Microliter Cell. This was followed by 1µl sample quantification. The ratio of absorbance at 260 to 280 reflects the purification of the RNA sample. Good-quality RNA is indicated by a 260:280 ratio of between 1.8 and 2.0.

2.6.4 RNA purification

Extracted RNA was treated with deoxyribonuclease I (DNase I) to eliminate any genomic DNA contamination. This was carried out by using a DNase I Kit (Thermo Fisher Scientific, 18047019) according to the manufacturer's instructions. Briefly, the RNA sample in 8µl of DNase-free water was mixed with 2µl of the master mix, which was formed by adding 1µl of 10x reaction buffer and 1µl of DNase (volume/reaction), and incubated at room temperature for 15 minutes, which was followed by the addition of 1µl DNase stop solution to each sample. The samples were incubated for 10 minutes at 70^{o}C and then placed on ice in preparation for the next reverse transcription step.

2.6.5 Reverse transcription

The cDNA was generated by using the high-capacity RNA-to-cDNA Kit (Applied Biosystems, P/N 4387406). On ice, 1µl of RNA in 9µl DNase/RNase-free water was prepared and mixed with 11µl of the master mix generated using the ingredients listed in Table 2-7. The mixtures were placed into the PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA) along with a negative control (RNA treated as indicated but with no addition of the reverse transcriptase buffer). The program was set to incubate the samples at 37°C for 1 hour and then heat up to 95°C and cool down to 4°C, being maintained indefinitely. This allows the reaction to stop. Generated cDNA was stored at -20°C for future use.

Table 2-7 RNA-to-cDNA Kit reverse transcription master mix ingredients.

Ingredients	Volume/reaction (µl)
2x reverse transcriptase buffer (includes dNTPs, random octamers, and Oligo dT-16)	10
20x reverse transcriptase enzyme (MuLV and RNase inhibitor protein)	1

2.6.6 Quantitative PCR (qPCR)

qPCR depends on the detection of PCR products made by reactions with a fluorescent molecule that reports an increase in the amount of DNA with a proportional increase in the fluorescent signal. With the use of SYBR Green Jumpstart™ Taq Readymix™ (Sigma, S4438) a master mix was prepared using the components listed in Table 2-8. The mix was created in PCR tubes (Alpha Laboratories, LW2340) in which 2µl of cDNA was mixed with 10µl of the master mix. The mixture was vortexed and then loaded into a 96-well plate in duplicates. In addition, water and cDNA (without the reverse transcriptase enzyme) negative controls were added to rule out possible contamination.

Table 2-8 qPCR condition.

qPCR program setting	Components	Volume/reaction (µl)
95°C 10 minutes } **40 times** **95°C 15 seconds** **60°C 1 minute** **Melt curve:** A melt curve at every 0.3°C from 60 to 95°C was measured for each experiment to confirm the specificity of PCR products.	**ddH$_2$O**	0.9
	SYBR Green JumpStart Taq ReadyMix (Sigma) (20mM Tris-HCL, pH 8.3, 100mM KCL, 7mM $MgCl_2$, 0.4mM each dNTP (dATP, dCTP, dGTP, TTP), stabilisers, 0.05 units/µl Taq DNA polymerase, JumpStart Taq antibody, and SYBR Green I)	5
	Internal reference dye	0.1
	Forward primer pairs (10µM)	1 (1µM)
	Reverse primer pairs (10µM)	1 (1µM)
	cDNA	2

An optically clear sealing sticker (Sarstedt, 95.1994) was used to seal the 96-well plate before it was quickly centrifuged using a plate spinner (MPS 1000 Mini PCR Plate Spinner) to ensure the movement of the added mixture to the bottom of the wells. The plate was inserted into the ABI StepOne Plus Real-Time PCR System (Applied Biosystems, 4376600) and the program for the thermal cycles was set as indicated in Table 2-8. The relative mRNA levels against the reference gene were calculated as $2^{-\Delta Ct}$, as previously described in (Kondaveeti, Reed et al. 2015). The primer pairs were purchased from Thermo Scientific, and GAPDH was used as a reference gene. Table 2-9 indicates the sequence of the forward and reverse primers used.

Table 2-9 Summary of primer pairs for qPCR.

Gene	Type	Melting Tm (°C)	Amplicon Size (bp)	Primer sequence (5'-3') (Forward and Reverse)
IGF-IR	Target	86.78	130	F: AGCCGATGTGTGAGAAGACC R: TGGCAGCACTCATTGTTCTC
IR-A	Target	85.15	120	F: TTCGGCCGGCGAATGCTGCT R: CCGAGTGGCCTGGGGACGA
IR-B	Target	87.70	122	F: AAAACCTCTTCAGGCACTGG R: GAGGAAGTGTTGGGGAAAGC
IR	Target	83.96	154	F: TGACAACGACCAGTGTGGAG R: GCAGCCGTGTGACTTACAGA
PPARγ	Target	81	143	F: GGTGGCCATCCGCATCT R: TGCTTTTGGCATACTCTGTGATCT
Adiponectin	Target	86	200	F: TCAGCATTCAGTGTGGGATTG R: GGTAAAGCGAATGGGCATGT
GLUT-4	Target	85.3	90	F: GCGGCGAAGATGAAAGAAC R: CTCCAGGCCGGAGTCAGA
GAPDH	Ref-erence	84.1	259	F: GATCATCAGCAATGCCTCCT R: TGTGGTCATGAGTCCTTCCA

2.7 Glucose uptake assay

2.7.1 Principle

Cellular metabolic activity and glucose uptake can be measured in cultured cells through the use of radiolabelled glucose analogue 2-deoxyglucose (2DG). When the 2DG is transferred into the cells it is phosphorylated by hexokinase (HK) to 2-deoxyglucose 6-phosphate (DG6P), which is a stable metabolite that is trapped and accumulated inside of the cell (Yamamoto, Ueda et al. 2011).

2.7.2 Buffers/reagents

10× calcium-free KREBS salts stock	500ml
1.36M NaCl	39.7g
47mM KCl	1.75g
125mM $MgSO_4.6H_2O$	12.7g

2× sodium phosphate buffer stock (stored in fridge)

10mM NaH_2PO_4 0.78g in 500ml
10mM Na_2HPO_4 0.71g in 500ml
Add 387ml of Na_2HPO_4 to 113ml NaH_2PO_4 *(will give a pH of 7.4, total of 500ml solution)*

50mM glucose: 0.180 of glucose in 10ml water

125mM $CaCl_2$ (fresh): 0.28g in 20ml H_2O

200mM $NaHCO_3$ (fresh): 0.168g in 10ml H_2O

25mM HEPES: Dissolve 1M stock solution 1 in 40 with sterile water.

Krebs–Ringer phosphate (KRP) buffer solution	25ml
10× Ca-free KREBS stock	2.5ml
125mM $CaCl_2$ (fresh)	0.25ml
2× sodium phosphate stock	12.5ml
200mM $NaHCO_3$ stock	0.25ml
HEPES 25mM	0.625ml

(adjust pH to 7.4 to make up to 25ml)

Radiolabelled glucose (for 1ml in KRP buffer)	1ml
50mM glucose	10µl
Tritiated 2 deoxyglucose (Amersham Pharmacia Biotech, Little Chalfont, UK)	5µCi
KRP buffer	985µl

2.7.3 Protocol

Cells were washed three times with PBS (room temperature), and then 900µl of glucose-free KRP buffer was added to each well and incubated for 15 minutes at 37^{o}C. Following this incubation, the plate was transferred into a 37^{o}C water bath so that the bases of the wells were submerged. The assay can be performed at room temperature, but the changes in glucose uptake observed will be much smaller and slower to occur. After 15 minutes' incubation with KRP buffer, the stimulus was added (e.g. 100nM insulin). Then, 100µl of radiolabelled 2-deoxyglucose (0.5µCi per well) was added to each well for 10 minutes.

Glucose transport was terminated by transferring the plate, removing the KRP buffer and washing the cells gently with ice-cold PBS. The cells were solubilised in 0.5ml 1% Triton X 100/PBS. Each solubilised cell suspension was mixed with 2ml of

scintillation fluid and counted on a β-Scintillation Counter (Beckman, High Wycombe, UK). Data were recorded as disintegrations per minute (DPM). Basal glucose uptake was measured without insulin or IGF-II stimulation.

2.8 Radioimmunoassay (RIA)

2.8.1 Principle

This competition assay uses radioactively-labelled peptide for detection. It measures radioactivity associated with immune complexes. Ligand in the samples (i.e. IGF-II) competes with iodinated tracer (IGF-II antigen) for binding to the antibody (anti-IGF-II). After excess tracer was washed away, γ-radiation emitted from the tracer bound to the antibody was detected and the concentration of the ligand in the samples was calculated from the standard curve. The principle is illustrated in Figure 2-9.

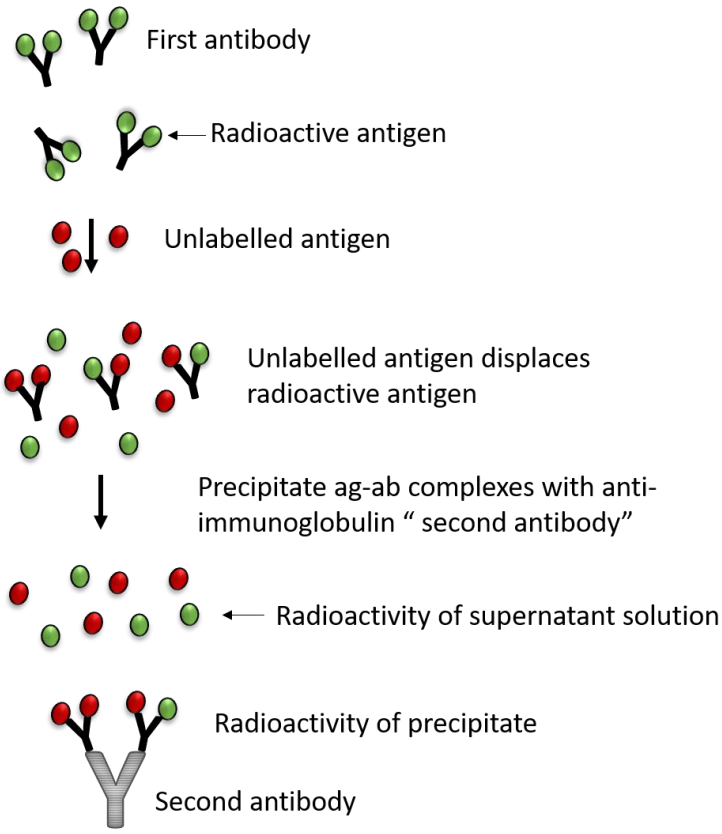


Figure 2-9 A schematic illustration showing the principle of RIA. ***Adapted from www.perkinelmer.co.uk*****.**

2.8.2 Equipment

- Gamma Counter (BERTHOLD, LB 2111)
- Vortexer (POTAMIXER DELUXE, Hook & Tucker Ltd.)
- Test tubes (11 x 64mm) (Alpha Laboratories, DA 3510)

2.8.3 Reagents

Table 2-10 Reagents used for radioimmunoassay.

Reagent	Description
Assay buffer	Formed by 0.2g Protamine sulphate (Sigma – P4020), 3.6g (0.03M) sodium dihydrogen phosphate (BDH – 301324Q), 0.2g (3mM) sodium azide

	(Sigma – S2002), 3.72g (10mM) EDTA (Sigma – E5134), and 500µl (0.05%) TWEEN 20 (Sigma – P5927). Added to 1L dH2O, adjusted to pH 7.5 and stored at 4°C.
Acetic acid solution	0.2875ml glacial acetic acid (BDH – 27013), 0.01g (0.01%) BSA (Sigma – A7888), and 50µl (0.05%) TWEEN 20 (Sigma – P5927). Added to 100ml of dH2O, aliquoted into 30ml and stored at -20°C.
8M formic acid	Formed by 33.92ml of neat formic acid added to 100ml of ddH2O, and 3–4 drops of TWEEN 20 were added to give approximately 0.05% TWEEN (stored at room temperature).
1M Tris base	Formed by adding 12.11g of Tris base to 100ml of ddH20, stored at room temperature.
Acetone	(BDH, Poole, UK)

2.8.4 Procedure

An extraction procedure is performed for IGF-I and -II measurements to release the ligands from their endogenous binding proteins. This step must proceed the RIA assay. It will help to avoid the reduction in the detection of IGF levels due to the competition of the binding proteins with the IGF antibody. 50µl of the sample (including the NHS control) were aliquoted into a 1.5ml Eppendorf. 25µl of the formic acid/TWEEN solution were added to each sample and vortexed. Using an acetone primed pipette tip, 175µl of acetone were added to each sample, remembering to prime the tip between each sample. The samples were immediately capped, vortexed and centrifuged for 30 minutes at 4ºC at an rcf of 3000g. In a fresh Eppendorf tube, 100µl

of supernatant were added and mixed with 100µl of Tris base. The serum samples were further diluted to 1:10 with assay buffer (30µl into 270µl). The conditioned media when analysed did not need dilution, so standards were supplemented with 50µl of acid/acetone/Tris base solution, in addition to 50µl of the unconditioned media to ensure that all conditions were maintained. Normal human serum (NHS) at a dilution of 1:80 (in assay buffer) was used as a positive control.

Recombinant human IGF standards were prepared with assay buffer to generate a standard curve with the following dilutions for IGF-I: 0, 0.25, 0.5, 1.0, 2.5, 5, 10, 25 & 50ng/ml, and for IGF-II: 0, 0.5, 1.0, 2.5, 5, 10, 25 & 50ng/ml.

Freshly made radioactive tracer with I^{-125} labelled IGF-I and IGF-II was prepared by adding 50µl of the labelled tracer to 25ml of the assay buffer. 50µl of the solution were added to a test tube and the number of counts was measured using a bench-top gamma counter. The number of counts needs to be between 10,000 and 15,000.

IGF-I RIA antiserum was a monoclonal antibody (Clone M23/ILG1-001, 5345-0304) diluted with assay buffer at 1:2000, and for IGF-II (W5D2) an in-house antibody was used at a 1:25,000 dilution.

Because RIA is very sensitive to residual binding protein, excess 500ng/ml IGF-I or 200ng/ml IGF-II was added to the assay respectively (i.e. IGF-I for the IGF-II assay, and *vice versa*) to completely remove any interference from residual binding proteins remaining after the extraction procedure. The peptides will not affect the levels of the measured IGFs because they will not be identified by the antibodies.

Tubes were labelled in duplicates for TC (total counts of the tracer), NSB (non-specific binding, which determines the level of iodinated peptide binding to the tube, usually

less than 3% of TC), maximum binding (around 40% of TC), and each assay standard. Tubes were labelled in triplicate for the samples and were set up as indicated in Table 2-15 for IGF-I and Table 2-16 for IGF-II.

Each tube was vortexed (except for TC tubes), covered with cling film and incubated at 4°C (in the fridge) overnight. 50µl of anti-mouse SAC-CEL (IDS Ltd., Bolden, UK) were added to each tube (except for the TC tubes) for IGF-I and -II RIA. All tubes were vortexed, covered with cling film and incubated for 30 minutes at 4°C. SAC-CEL is a solid-phase (covalent-coupled) secondary antibody coated cellulose suspension that enables the pull-down of primary antibodies in solution after centrifugation and was used with continuous stirring. 1ml of distilled water was added to each tube and tubes were centrifuged at 2000g at 4°C for 30 minutes (except for the TC tubes). The generated supernatant was aspirated and, finally, each tube was counted using the Gamma Counter. The concentration of IGF-I and IGF-II ligand was calculated automatically from the standard curve by a pre-set computer program.

Table 2-11 IGF-I RIA reagent preparation.

	Assay buffer (µl)	**Sample (µl)**	**Standards (µl)**	**Tracer (µl)**	**Antibody (µl)**	**IGF-II (µl)** (excess)
TC	-	-	-	50	-	-
NSB	400	-	-	50	-	50
Maximum binding	350	-	-	50	50	50
Standards	300	-	50	50	50	50
Samples	300	50	-	50	50	50

Table 2-12 IGF-II RIA reagent preparation.

	Assay buffer (µl)	**Sample (µl)**	**Standards (µl)**	**Tracer (µl)**	**Antibody (µl)**	**IGF-I (µl)** (excess)
TC	-	-	-	50	-	-
NSB	400	-	-	50	-	50
Maximum binding	350	-	-	50	50	50
Standards	300	-	50	50	50	50
Samples	300	50		50	50	50

2.8.5 IGFBP-3 radioimmunoassay

The method for IGFBP-3 quantification in conditioned media is identical to IGF-I and IGF-II RIA quantification, as explained previously (2.8.4). Standards were prepared from normal human serum diluted in 100mM acetic acid to generate a standard curve of 0, 6.6, 13, 33, 66, 131, 312 and 624ng/ml. Acetic acid is helpful in keeping IGFBP-3 from sticking to the plastic tubes. 50µl unconditioned medium were added to the standards (except for TC tubes), whereas a 50mM acetic acid solution (described in Table 2-10) was added to the samples to allow for continuity between standards and samples.

Non-glycosylated I^{-125} labelled IGFBP-3 tracer was used and diluted in assay buffer to give a reading of 15,000cpm/50µl. In-house antiserum (SCH5) was used and diluted at 1:2000 in assay buffer. The tubes were set-up in a similar manner to those in the IGF-I/IGF-II assay, and samples were arranged in triplicate and prepared as indicated in Table 2-13. NHS was used as a positive control at 1:100 and 1:80 dilutions in assay buffer.

Table 2-13 IGFBP-3 RIA reagent preparation.

	Assay buffer (µl)	Acetic acid (µl)	Sample (µl)	Standards (µl)	Tracer (µl)	Antibody (µl)	Media (µl)
TC	-	-	-	-	50	-	-
NSB	350	50	-	-	50	-	50
Maximum binding	300	50	-	-	50	50	50
Standards	300	-	-	50	50	50	50
Samples	300	50	50	-	50	50	-

Tubes were vortexed, incubated at 4°C overnight, and 50µl of anti-rabbit SAC-CEL (IDS Ltd., AASAC1, UK) were added to each tube (except for the TC tubes). Tubes were vortexed, covered with cling film and incubated at 40°C for 30 minutes. 1ml of distilled water was added to each tube (except for the TC tubes), and the tubes were centrifuged at an rcf of 2000g at 4°C for 30 minutes.

The resulting supernatant was aspirated and the radioactive pellets were counted on a gamma counter. IGFBP-3 levels in the samples were automatically calculated from the standard curve.

2.9 Microscopy

Inverted microscopes (Nikon Eclipse TS 100 and Zeiss AXiO Vert A1) were used for counting and imaging cells. Motic Image and Q-Capture Pro7 software were used for capturing images.

2.10 Statistical analysis

SPSS 12.0.1 (SPSS Inc., Chicago, USA) for Windows was used for statistical analysis. Results were represented as the mean ± standard error (SEM). The number of biopsies used was indicated for each experiment. Analysis of variance (ANOVA) was used for multiple comparisons, followed by a least significant differences (LSD) post-hoc test. Pearson's correlation coefficients (and subsequent linear regression) were used to assess IGF-II correlations with fat mass in the ALSPAC data. Non-parametric data were log-transformed to remove any skew.

A value of $p \leq 0.05$ was considered statistically significant.

Chapter 3 : Characterization of subcutaneous and visceral adipose tissue from pre-pubertal children

3.1 Introduction

Obesity is a health concern of epidemic proportions that is increasing worldwide. The negative consequences of obesity on health have mainly been reported in studies of adults: however, during recent years, it is clear that childhood obesity is markedly increasing and is also associated with a rise in morbidity and mortality (Lobstein, Jackson-Leach et al. 2015). An excess accumulation of adipose tissue results in obesity. The adipocyte has a critical role, normally functioning to buffer changes in availability of metabolic fuels, storing energy in the form of lipid at times of plenty and then providing a source of free fatty acids (FFA) to supply energy during times when food is limited (Frayn 2002). It is now clear that adipose tissue is also a very active endocrine organ, secreting and responding to many hormones and cytokines that play an important role in the regulation of appetite, fuel partitioning and utilization. The distribution of body fat has greater importance for the development of obesity-related morbidities than the simple extent of excess adipose tissue. Elimination of a large proportion of subcutaneous fat by liposuction has little effect on insulin sensitivity (Klein, Fontana et al. 2004), whereas removal of visceral fat by omentectomy significantly improves insulin sensitivity independent of change in total body weight (Thörne, Lönnqvist et al. 2002). It has also become apparent that adipocytes from different anatomical sites serve distinct functional roles. The visceral adipocytes have a specialized function due to their anatomical location with venous drainage via the portal system; they are more metabolically active and rapidly release nutrients during conditions of stress, directly providing FFA as substrates for hepatic glucose production and lipoprotein metabolism. This evolved specialist function for

providing alternative metabolic fuel at times of stress may however be maladaptive during times of prolonged positive energy balance, which throughout most of evolution would have been extremely rare. A western lifestyle with regular snacks and energy-dense foods however results in humans being in a postprandial state for 16-18 hours per day (Parks 2002). In line with this specialized function, a number of distinct characteristics have been described for visceral adipocytes including higher rates of catecholamine-induced lipolysis (Rebuffe-Scrive, Andersson et al. 1989) and higher levels of β–adrenergic receptors (Arner, Hellström et al. 1990). In addition, regional differences have been reported for IGF production and receptor distribution in animal studies (Tchoukalova, Nathanielsz et al. 2009). Interestingly, changes in the IGF system have been described in obese children (Saitoh, Kamoda et al. 1998, Ballerini, Ropelato et al. 2004) although far less is known about site-specific differences in adipocytes in children.

Experimental adipocyte models are very limited; there are no available human adipocyte cell lines and the majority of work to date has been undertaken in a murine cell line, 3T3-L1. While preadipocytes can proliferate in culture, those derived from adult humans will no longer differentiate into mature adipocytes following just one or two passages in culture *ex vivo*. The depot-specific differences in adipocyte function, that are so significant for human morbidities are very different and much less marked in rodents (Arner 2005), and the functional distinctions of visceral adipocytes therefore cannot be examined in murine cell lines. In our laboratory, we have overcome these limitations and are able to show that in contrast to cells from adult subjects, preadipocytes from children retain the ability to effectively differentiate following several passages in culture *ex vivo* enabling significant numbers of cells to be grown for experimentation (Grohmann, Sabin et al. 2005). We hypothesis that IGF-

II is predominantly secreted by adipose cells, and IGFs receptor levels will differ between visceral and subcutaneous preadipocytes and differentiated adipocytes; showing a higher level of insulin receptor isoform A in visceral cells in comparison to subcutaneous cells.

3.2 Aims and objectives

In this chapter, we aim to highlight the characteristic differences in subcutaneous and visceral preadipocytes and adipocytes from children to establish a fundamental foundation for future understanding and experimentation. This will be conducted by:

- Culture and differentiate subcutaneous and visceral fat biopsies taken from pre-pubertal children
- Characterize fat-depot differences between subcutaneous and visceral preadipocytes in terms of:

a. Secretory levels of IGFs
b. Genetic expression and protein abundance of glucose transporters (GLUT1, GLUT4) in preadipocytes and adipocytes
c. Basal- and insulin-stimulated glucose uptake
d. IGF receptor expression (IGF-IR, IR, insulin receptor isoforms)

3.3 Material and methods

3.3.1 3T3-L1 cell line culture and differentiation

3T3-L1 preadipocytes were grown in T75 flasks to 70% confluency, and then plated into 6-well plates and induced to differentiate over 7 days using differentiation media, as described in section (2.2.4)

3.3.2 Child fat biopsy preparation, culture and differentiation

Paired biopsies from subcutaneous and visceral fat were prepared as described in the methods section (2.2.5). Briefly, fat biopsies were washed with 10ml of HBSS, dissected into small pieces (1mm3) and digested using type II collagenase (1 mg/ml) in HBSS at 37°C using a shaking water bath for 1 hour. Preadipocytes were separated from the stromal vascular compartment by centrifuging for 3 minutes at 80g. The sedimented cells were cultured in a gelatin-coated T75 flask after resuspension with 10 ml of DMEM/Ham's F12 medium (1:1, v/v) supplemented with 20% FBS, 2 mM glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Cells were incubated at 37°C in a humidified atmosphere of 5% CO2, and the medium was changed every 2 days until confluency was reached (7-14 days). Cells were washed with PBS and detached from flasks using 1 ml trypsin and incubated for 5 minutes. Following incubation, trypsin was deactivated using 5 ml of growth media and the cell suspension was centrifuged at 80g for 5 minutes. For differentiation, cells were plated on a gelatin-coated 6-well plate with a seeding density of 0.2 X 10^6 and were differentiated for 14 days using the differentiation media described in the methods section (2.2.2). To maintain consistency, experiments were only performed using cell passages 3–5.

3.3.3 Western immunoblotting

Preadipocyte differentiation markers, glucose transporters and receptor protein abundance were visualized using SDS-PAGE and western blotting. Protocol and

antibody concentrations are described in section (2.4). GAPDH was used as a reference protein. Quantification of band intensities was determined by Image Lab 1.46r software.

3.3.4 RNA extraction and RNA- cDNA reverse transcription

RNA was extracted by Trizol reagent (Invitrogen) as outlined in section (2.6.2) and 1 µg of RNA was reversed to cDNA using a high capacity RNA-to-cDNA kit (Applied Biosystems) as described in section (2.6.5)

3.3.5 Quantitative Polymerase Chain Reaction (qPCR)

mRNA genetic expression of differentiation markers, glucose transporters, insulin and IGF-I receptors and insulin receptor isoforms were performed using SYBR green-based qPCR. The PCR reaction was analysed using StepOnePlus and relative mRNA levels were determined using the $2^{-\Delta Ct}$ method after normalization to the GAPDH reference gene. For protocol details and primer sequencing refer to section (2.6.6)

3.3.6 Glucose uptake

[^{3}H]2-deoxyglucose uptake was performed for basal- and insulin-stimulated glucose uptake, ten µg of insulin (100nM) was added for 15 minutes and glucose uptake was quantified using a β-Scintillation Counter. Data are presented as disintegrations per minute (DPM). Further protocol details are mentioned in section (2.7)

3.3.7 Oil red O Triglyceride staining

Cultured preadipocytes were washed twice by PBS before being fixed in 10% formalin, with Oil red O (ORO) staining being performed on day 0 and day 7 post-differentiation for the 3T3-L1 cell line and on day 0 and day 14 post differentiation for human cultures; further details of ORO is described in section (2.3). Quantification

of the ORO stain was carried out using spectrophotometry (FLUOstar OPTIMA, BMG LABTECH) at 490nm.

3.3.8 Radioimmunoassay (RIA)

Conditioned media levels of IGF-I and IGF-II were assessed using a radioimmunoassay as described in section (2.8).

3.3.9 Statistical analysis

Student's t-tests were used for 3T3-L1cells analysis (at day 0 and 7 of differentiation). One-way ANOVA followed by a least significant difference (LSD) post-hoc test was used for multiple group comparisons (subcutaneous and visceral) pre and post-differentiation (day 14). SPSS 12.0.1 for Windows were used for analysing data with a significant statistical difference at $P<0.05$.

3.4 Results

3.4.1 Optimizing of preadipocyte differentiation using 3T3-L1 cell lines

The process of preadipocyte differentiation has been studied in detail for the past 20 years. It can be described as the ability of committed preadipocytes to change into adipocytes by undergoing growth arrest and is followed by terminal differentiation. This process is associated with an increase in specific mature adipocyte genes and lipid-processing enzymes and it is controlled by many transcription factors such as PPAR-γ and C/EBP (Altiok, Xu et al. 1997). The most extensively used experimental model to study fat cells is the 3T3-L1 murine cell line, which we decided to use to master the cell culture and differentiation techniques. Figure (3-1) illustrates the morphological changes of the 3T3-L1 preadipocytes from fibroblast-like cells to adipocytes containing spherical shaped lipid droplets. At 72 hours, the cells were 70-80 % confluent, and 100% confluent at 96 hours. Following the introduction of differentiation media two days post-confluency, morphological changes from fibroblastic cells to rounded adipocytes appeared to start from day 2 of differentiation.

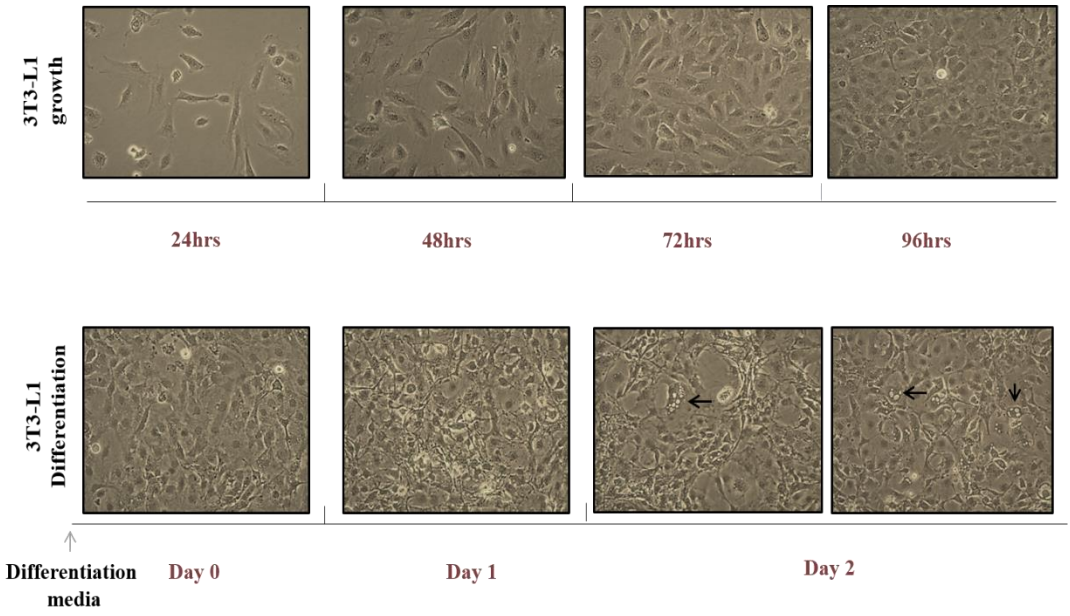


Figure 3-1 Phase contrast microscopy showing the preadipocyte growth and differentiation process in 3T3-L1 cell lines.

Morphological change of preadipocytes from fibroblastic cell to adipocyte fat-containing cells (arrows); 3T3-L1 confluency was achieved after 96 hours and fat accumulation started at day 2 post-differentiation. Magnification X10.

With adipocyte maturation, triglycerides start to accumulate within the cells. This adipogenic potential can be assessed using Oil red O staining to confirm the morphological changes and evaluate the differentiation process (Ramirez-Zacarias, Castro-Munozledo et al. 1992). Figure (3-2-a) shows the morphological changes of preadipocytes (day 0) and the increase in Oil red O stain deposition after differentiation (day 7). There is a 40-fold increase in Oil red O spectrophotometry absorbance (490nm) in comparison to preadipocytes (adipocytes 40.35, preadipocytes 0.335; $P<0.01$), Figure (3-2-b).

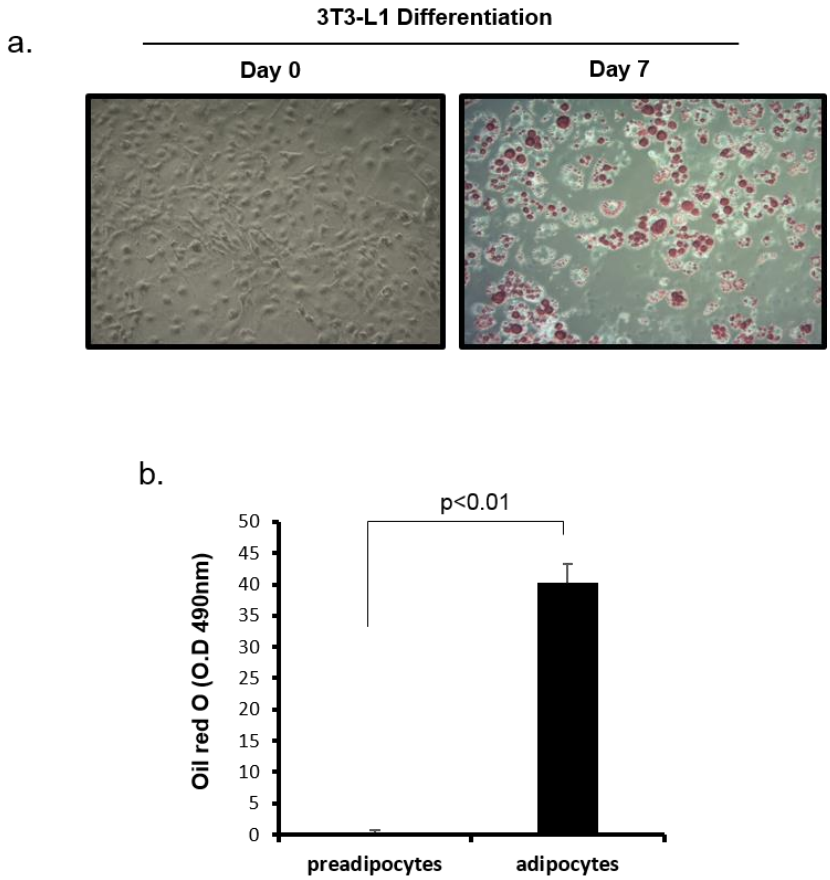


Figure 3-2 3T3-L1 cell line differentiation assessment using Oil red O stain.

(a) Oil red O staining of 3T3-L1 preadipocytes at experimental day 0 and day 7 showing red colour triglycerides accumulation at day 7 (Magnification X10) (b) Oil red O spectrophotometry absorbance analysis indicating an increased fat deposition with differentiation. Data is presented as mean ± SEM of two experimental repeats performed in triplicate.

To further confirm 3T3-L1 preadipocyte differentiation protein abundance of peroxisome proliferator-activated receptor gamma (PPAR-γ), which is known to increase with differentiation, was determined using western blotting. This marker acts as a trans-activator of adipocyte genes and is involved in the cell growth arrest that is needed to start differentiation. The protein abundance of PPAR-γ increased gradually with differentiation; in comparison to day 0 of differentiation there was a 6.2-fold

increase in PPAR-γ at day 5 ($p<0.01$) and a 12-fold increase at day 7 of differentiation ($p<0.001$). illustrated in (Figure 3-3).

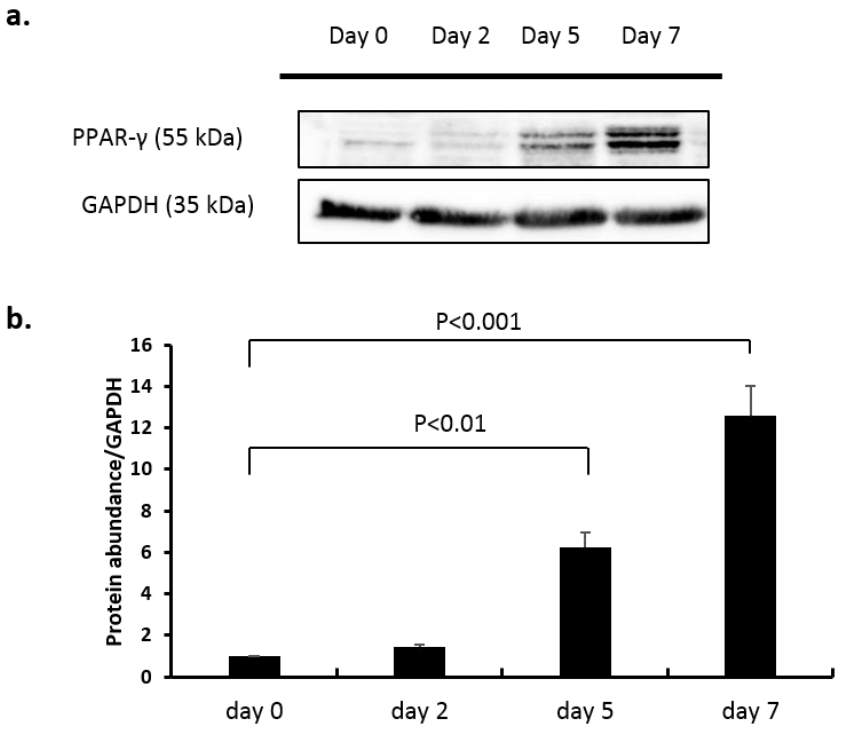


Figure 3-3 PPAR-γ differentiation marker protein abundance in 3T3-L1 cell lines at day 0, 2, 5 and 7 of differentiation.

(a) Western immunoblot illustrating PPAR-γ expression increase with differentiation; GAPDH was used as a loading control protein (b) image J analysis of the western blot (a) The graphs represent the mean ± SEM of two independent repeats each conducted in triplicate.

3.4.2. Data relating to the prepubertal children that participated in subcutaneous and visceral fat biopsy collections

Samples were obtained from young children admitted to a regional children's hospital for elective surgery. The study was approved by the NRES Committee South West – Exeter (REC reference: 14/SW/0109). An invitation to the study and a written information sheet was sent by post along with the surgery admission letter to all

potential subjects. Parents/legal guardians were offered a verbal explanation of the study on the morning of admission for surgery and consent was obtained from the families of 20 participants. Further details about ethical approval, information sheets and consent forms are provided in the appendix section (9.1-9.4).

Children recruited were of normal weight, and were admitted to the Bristol Royal Hospital for Children for routine renal surgery (non-malignant, non-septic operations). Fat tissue samples were collected by two Consultant Paediatric Surgeons during the operation. Subcutaneous and intra-abdominal peri-nephric (visceral) fat biopsies (0.2-0.5g) were collected and transferred immediately to the laboratory. Clinical data for the participants are shown in Table 3-1.

Table 3-1 Clinical data of the 20 children who participated in fat biopsy collection.

Biopsy number	Sex	Age (years)	Type of operation	BMI (kg/m^2)	BMI SDS
1	Male	6	Pyeloplasty	16.1	-0.2
2	Male	<1	Pyeloplasty	14.1	-1.6
3	Female	4	Hemi-nephrectomy	16.7	+0.14
4	Female	2	Pyeloplasty	15.3	-0.8
5	Male	3	Pyeloplasty	15.1	-0.9
6	Male	1	Pyeloplasty	16.6	+0.07
7	Male	3	Nephrectomy	16.2	-0.2
8	Female	<1	Hemi-nephrectomy	18.3	+1.2
9	Male	<1	Pyeloplasty	16.6	+0.07
10	Female	<1	Pyeloplasty	18.9	+1.6
11	Male	4	Pyeloplasty	16	-0.3
12	Male	5	Pyeloplasty	16.5	0
13	Male	7	Pyeloplasty	16.5	0
14	Female	2	Hemi-nephrectomy	17.2	+0.4
15	Male	2	Pyeloplasty	15.2	-0.9
16	Female	7	Hemi-nephrectomy	17.2	+0.4
17	Male	2	Nephrectomy	16.7	+0.1
18	Female	<1	Nephrectomy	18.8	+1.4
19	Male	3	Pyeloplasty	17	+0.3
20	Female	1	Nephrectomy	18.4	+1.3

3.4.3 Differentiation of human subcutaneous and visceral fat biopsies

Regional, depot-specific differences in adipocyte function are likely significant for human metabolic health. As these functional distinctions cannot be examined in murine cell lines we used a culturing system of preadipocytes obtained from children.

Paired subcutaneous and visceral fat biopsies were cultured and differentiated for 14 days using the methods described in section (2.2.5).

3.4.3.1 Assessment of human subcutaneous and visceral fat culture differentiation using Oil red O staining

After optimization of adipocyte Oil red O staining using the 3T3-L1 cell line, we used Oil red O staining to evaluate the differentiation of children's subcutaneous and visceral fat cultures. Following fat biopsy preparation, culture and differentiation as described in the methods section, Oil red O stain assessment was performed on preadipocytes (day 0) and adipocytes (day 14) post differentiation. Figure (3-4 a) illustrates the morphological changes observed in subcutaneous and visceral preadipocytes from fibroblastic-like cells to fat-containing differentiated adipocytes stained with Oil red O triglycerides. Figure (3-4 b) represents the spectrophotometry absorbance quantification; Oil red O stain was undetectable for subcutaneous and visceral preadipocytes but there was a 18.5-fold increase for subcutaneous adipocytes ($P<0.001$) and a 16.5-fold increase for visceral adipocytes ($P<0.001$) in Oil red O staining absorbance in comparison to preadipocytes: however there were no significant differences in fat deposition between the two fat depots.

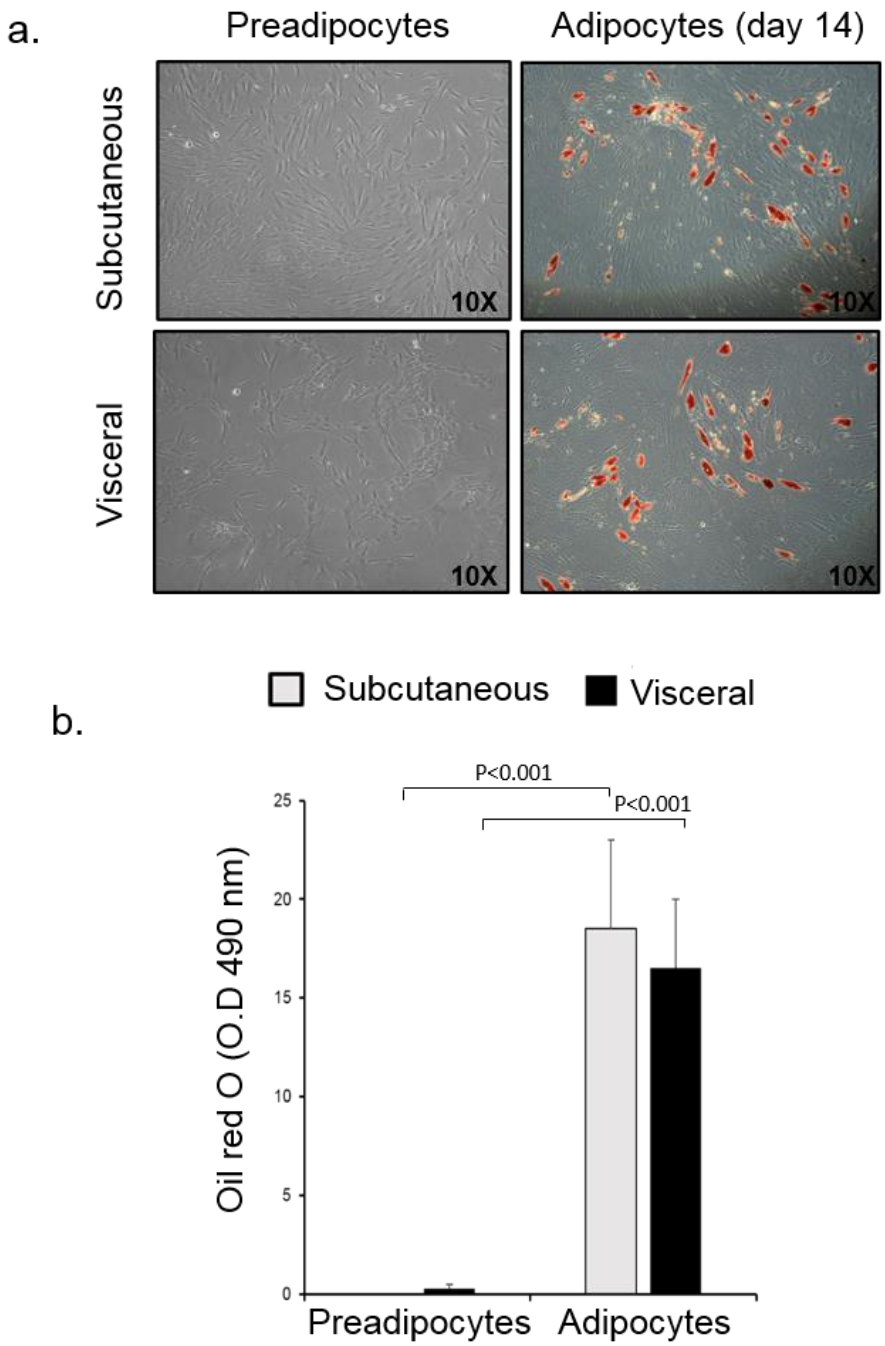


Figure 3-4 Characterization of subcutaneous and visceral preadipocyte fat biopsies from prepubertal children.

(a) Photomicrographs of human subcutaneous and visceral preadipocytes (day 0) displaying a fibroblastic morphology and differentiated adipocytes (day 14) stained with Oil red O. Magnification at (X10). (b) Quantitative absorbance analysis of Oil red O staining showing a significant increase in fat deposition in mature adipocytes in comparison with preadipocytes. The data are represented as mean ± SEM from 4 biopsies conducted in triplicate.

3.4.3.2 Assessment of subcutaneous and visceral differentiation markers using quantitative polymerase chain reaction (qPCR)

To further confirm the preadipocyte differentiation process, mRNA expression of the preadipocyte differentiation markers PPAR-γ and adiponectin, were analyzed. Trizol RNA extraction of preadipocytes (day 0) and adipocytes (day 14) was performed followed by qPCR as described in the methods section (2.6). The mRNA expression of both differentiation markers significantly increased in mature adipocytes (day 14) in comparison to preadipocytes (day 0) in both fat depots. Figure (3-5a) shows the increase in PPAR-γ in subcutaneous adipocytes (20.2 vs.1; $p<0.001$) and in visceral adipocytes (37.7 vs.2. 1; $P<0.001$) in comparison to preadipocytes. Figure (3-5b) shows the mRNA expression of adiponectin in subcutaneous adipocytes (14533 Vs.1; $P<0.001$) and in visceral adipocytes (23575 vs.1.8; $P<0.001$) vs. preadipocytes. A difference in PPAR-γ and adiponectin mRNA expression in visceral adipocytes in comparison to subcutaneous adipocytes was seen at day 14 of differentiation.

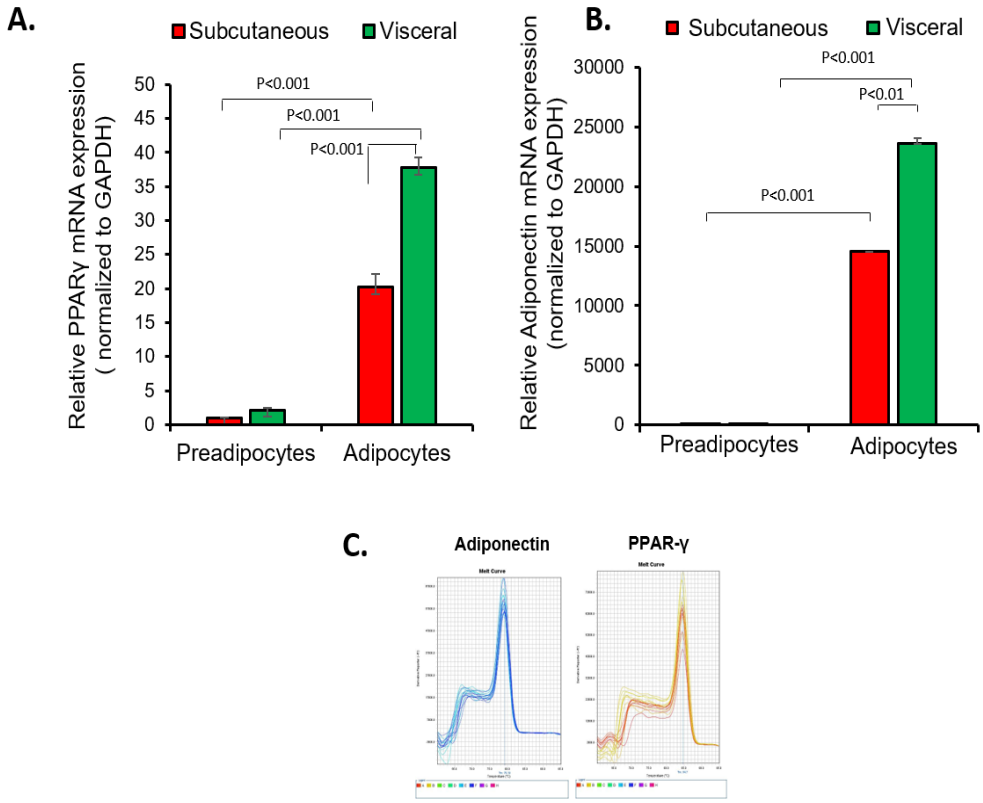


Figure 3-5 Relative mRNA expression of preadipocyte differentiation markers in subcutaneous and visceral preadipocytes and adipocytes obtained from pre-pubertal children.

mRNA expression of (A) PPAR-γ and (B) adiponectin quantified using SYBR green-based qPCR indicating an increase of mRNA expression with differentiation. (C) melting curves for PPAR-γ and adiponectin. GAPDH was used as reference gene. Data are expressed as mean ± SEM of duplicate runs (N=4).

3.4.3.3 Assessment of subcutaneous and visceral differentiation markers using western immunoblotting

This assessment determined whether the protein abundance of preadipocyte differentiation markers corresponded to mRNA expression. Western blotting was performed using specific PPAR-γ and adiponectin antibodies. At day 0 and 14 post-differentiation adipocyte whole cell lysate extracts were collected. As anticipated, a

significant increase in the differentiation marker PPAR-γ (Figure 3-6 b) in subcutaneous (adipocytes 2.7 Vs. 1 preadipocytes; p<0.01) and visceral (adipocytes 2.1 Vs.0.8 preadipocytes; P<0.01) was found. A similar increase was seen in adiponectin protein abundance (Figure 3-6c) with differentiation in subcutaneous (3.4 Vs. 1; P<0.001) and visceral (4.1 vs. 0.9; P<0.001).

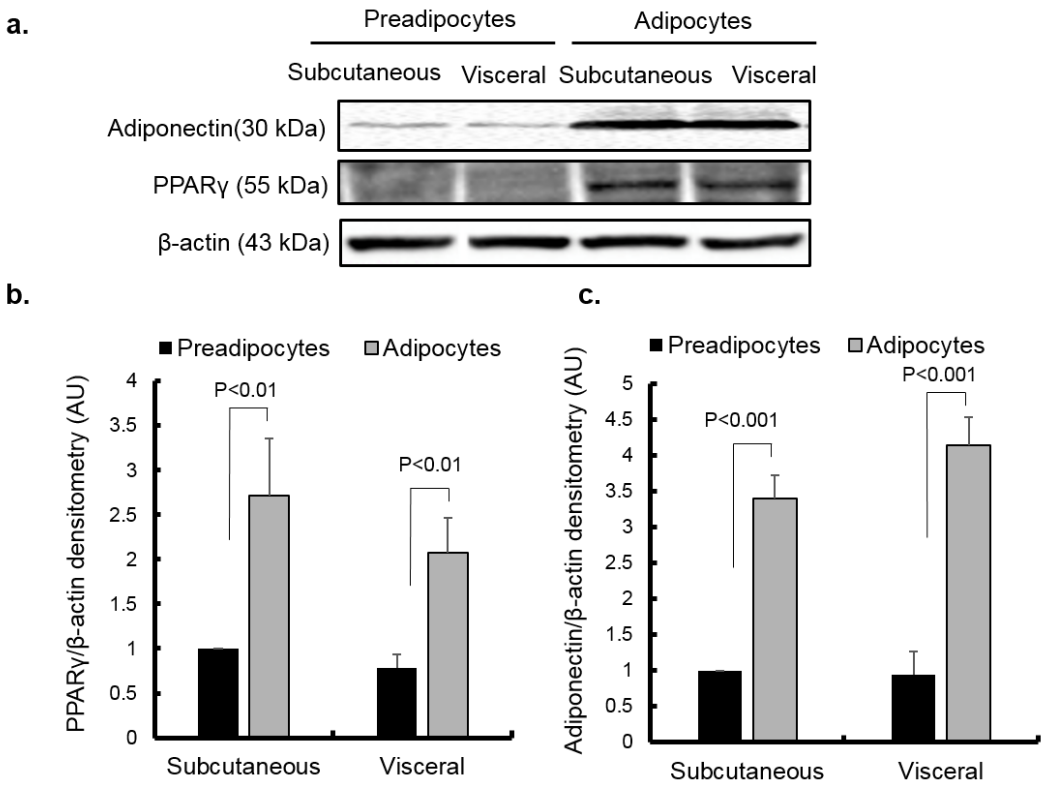


Figure 3-6 Protein abundance of preadipocyte differentiation markers in subcutaneous and visceral cutures obtained from pre-pubertal children.

Densitometry of the differentiation marker PPAR-γ (b) showing increase in protein abundance with differentiation in subcutaneous and visceral preadipocytes and adipocytes (day 14) (c) Densitometry qualification of adiponectin protein abundance with differentiation. β-action was used as a loading control. Data are expressed as mean ± SEM (N=4).

3.4.4 Fat depot differences in IGF-I and IGF-II local tissue production

Active adipocytes, in addition to their role in fat storage, are a secretory endocrine cell producing many hormones. To further asses the functionality of our fat cultures and address our future aim to study the role of IGFs in adipocyte regulation, we examined basal secretory levels of IGF-I and IGF-II from subcutaneous and visceral adipocytes. The levels of IGF-I and IGF-II in the media were measured after 24 hrs of culture in 5mM glucose-containing serum-free media using radioimmunoassay. Our results showed a significant predominance in IGF-II secretion over IGF-I from the two fat depots; almost a five-fold increase of IGF-II (4.86) secreted from visceral adipocytes in comparison to IGF-I (1.079) ($p<0.001$) and a 4.6-fold significant increase in subcutaneous IGF-II secretion in comparison to IGF-I (1.2) ($p<0.001$). There was no significant difference between the two-fat depot in IGF-II or IGF-I secretion (Figure 3-7).

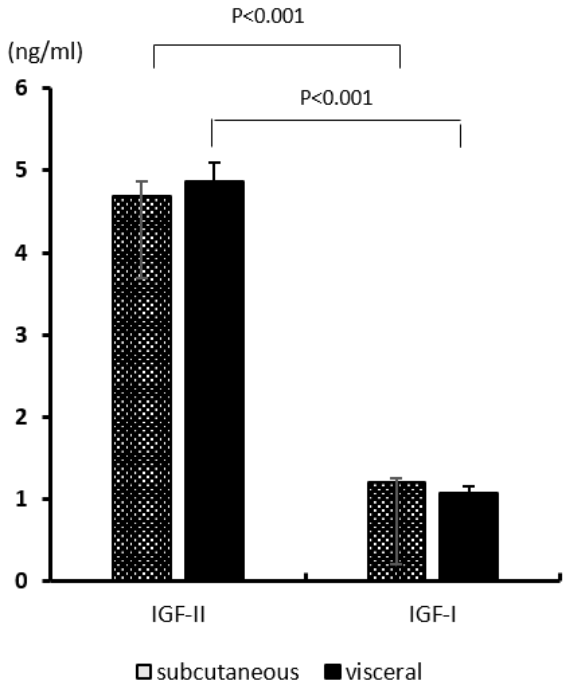


Figure 3-7 Secretory media levels of IGF-II and IGF-I from visceral and subcutaneous primary cell cultures obtained from pre-pubertal children.

IGF-II is predominantly secreted by mature adipocytes from children's primary cultures. IGF-II was measured using radioimmunoassay. Preadipocytes were cultured and differentiated into mature adipocytes for 14 days in normal glucose differentiation media (5mM). Data are presented as mean ± SEM of 3 repeats in triplicate and ANOVA was used to verify statistical significance (N=3).

3.4.5 Effect of hyperglycaemia on fat depot differences in IGF-I and IGF-II secretion

IGFs are nutritionally regulated and IGF secretory levels can be influenced by glucose concentrations (D'Esposito, Passaretti et al. 2012). To determine whether this applies to our model and to examine this phenomenon in a fat-depot context, we studied changes in adipocyte secretory levels of IGF-I and IGF-II. Media were collected after 24 hours' culture in 5mM or 25mM glucose-containing serum-free media. There was no significant difference between subcutaneous or visceral IGF-I secretion in normal and high glucose media (Figure 3-8).

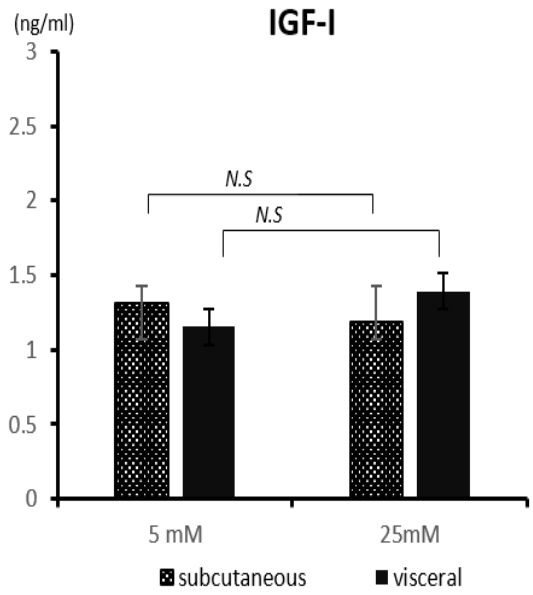


Figure 3-8 IGF-I secretion from subcutaneous and visceral adipocytes in different glucose conditions.

Preadipocytes were differentiated for 14 days then serum starved for 24 hours in normal (5mM) or high (25mM) glucose serum-free media. Media were collected, and RIA was performed using 3 different biopsies run in triplicate. Data are presented as mean ± SEM and ANOVA were used to verify statistical significance (N=3).

Similarly, there was no significant change in IGF-II secretion in high glucose (25mM) from visceral and subcutaneous adipocytes in comparison to normal glucose (5mM). Also, there was no difference in IGF-II production between the fat depots in high glucose conditions. (Figure 3-9).

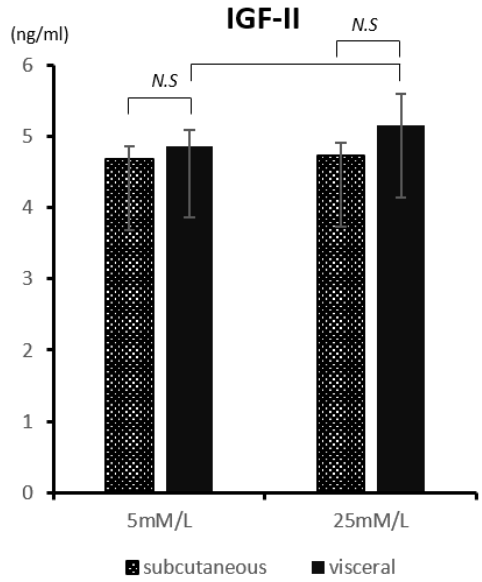


Figure 3-9 Effect of glucose concentration on IGF-II secretion from visceral and subcutaneous adipocytes.

IGF-II secretory levels were measured using radioimmunoassay (RIA). Media were collected after 24 hours' culture in normal (5mM) or high glucose (25mM) serum-free media. Data are presented as mean ± SEM of 3 experimental repeats in triplicate. ANOVA was used to verify statistical significance (N=3).

3.4.6 Characterisation of glucose transporters 1 and 4 (GLUT1, GLUT4) with differentiation in subcutaneous and visceral fat

The process of preadipocyte differentiation requires increased transcription of certain genes and expression of specific proteins such as the insulin-sensitive glucose transporter GLUT4. We investigated the change in glucose transporter expression and protein abundance in subcutaneous and visceral preadipocytes and differentiated adipocytes collected on day 14 post differentiation. Figure (3-10) illustrates the change in protein abundance of GLUT4; there was a significant increase in GLUT4 abundance with differentiation for both fat depots. Subcutaneous adipocytes have higher

expression of GLUT4 in comparison to preadipocytes (1.73 to 1; P<0.001). Also, visceral adipocytes have a significantly higher abundance than preadipocytes (2.8 to 1.3: P<0.001). However, there was no significant difference between subcutaneous and visceral GLUT4 expression in preadipocytes. The depot differences in GLUT4 abundance become more obvious with differentiation, with visceral adipocytes found to have a significantly higher expression in comparison to subcutaneous adipocytes (P<0.01).

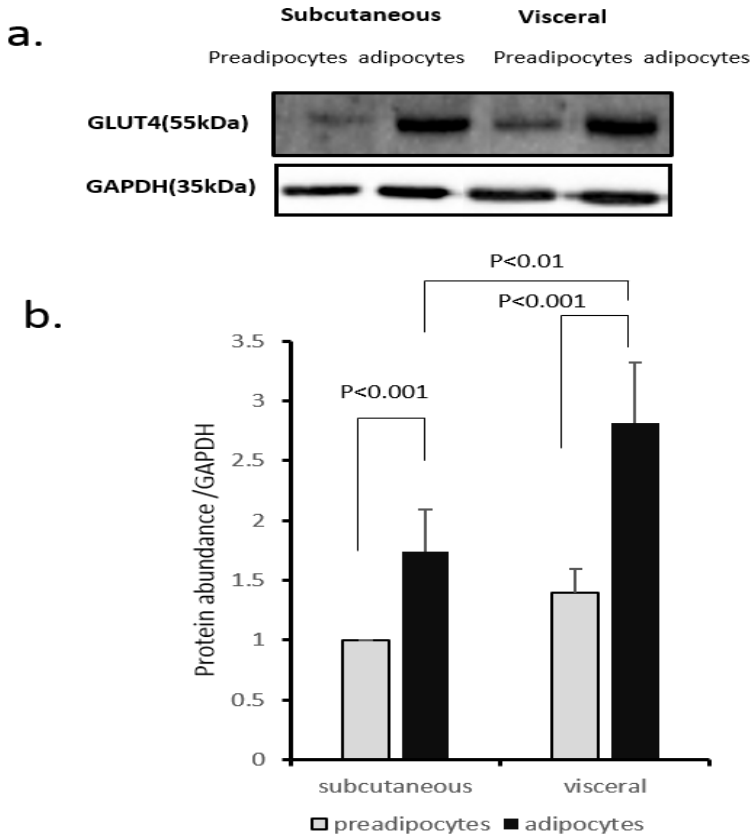


Figure 3-10 Fat depot protein abundance of GLUT4 in preadipocytes and adipocytes.

(a) Western blot of subcutaneous and visceral preadipocytes and adipocytes showing the increase of GLUT4 abundance with differentiation. (b) Semi-dosimetry quantification of western blot (a) The data represent mean ± SEM of 3 experimental repeats in triplicate (N=3).

In contrast to GLUT4, GLUT1 abundance decreased with differentiation as expected because adipocytes are more insulin dependent. Visceral preadipocyte abundance was (1.5 vs. 0.5: P<0.05) in comparison to adipocytes. Subcutaneous adipocytes also showed a decrease in GLUT1 abundance, but this did not reach statistical significance (Figure 3-11). A similar pattern of genetic expression of GLUT4 and GLUT1 was also seen and confirmed using qPCR (Figure 3-12).

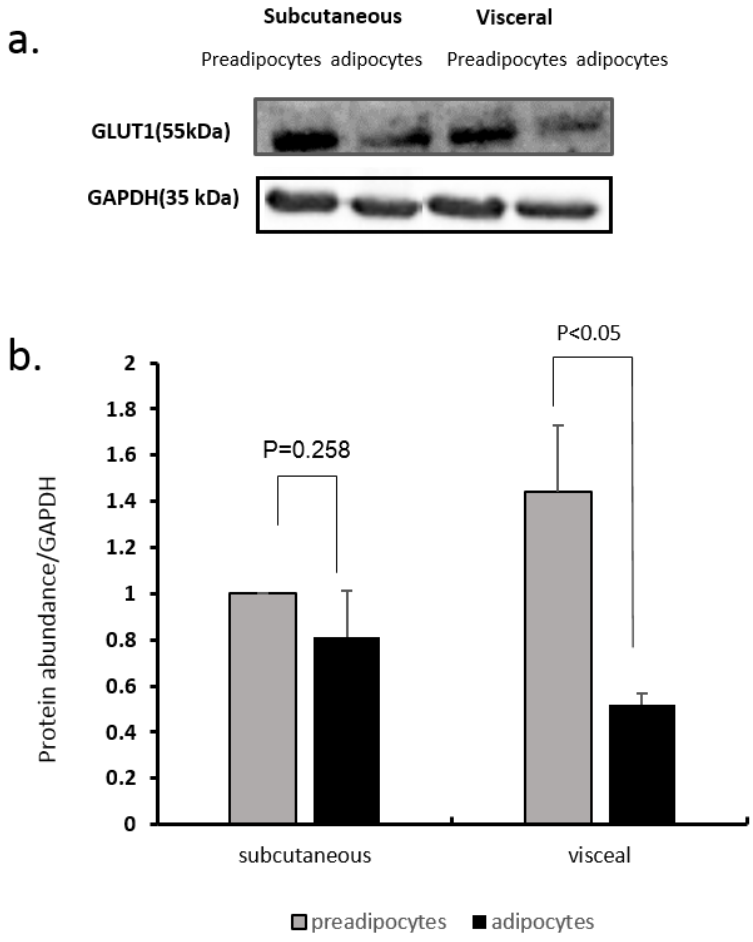


Figure 3-11 Characterization of GLUT1 protein abundance with differentiation.

(a) Western blot of GLUT1 in subcutaneous and visceral preadipocytes and adipocytes (b) Semi-quantitative analysis of (a) showing a decrease of GLUT1 abundance with

differentiation. GAPDH was used as a reference protein. Data are expressed as mean ± SEM of three experimental repeats in triplicate. (N=3)

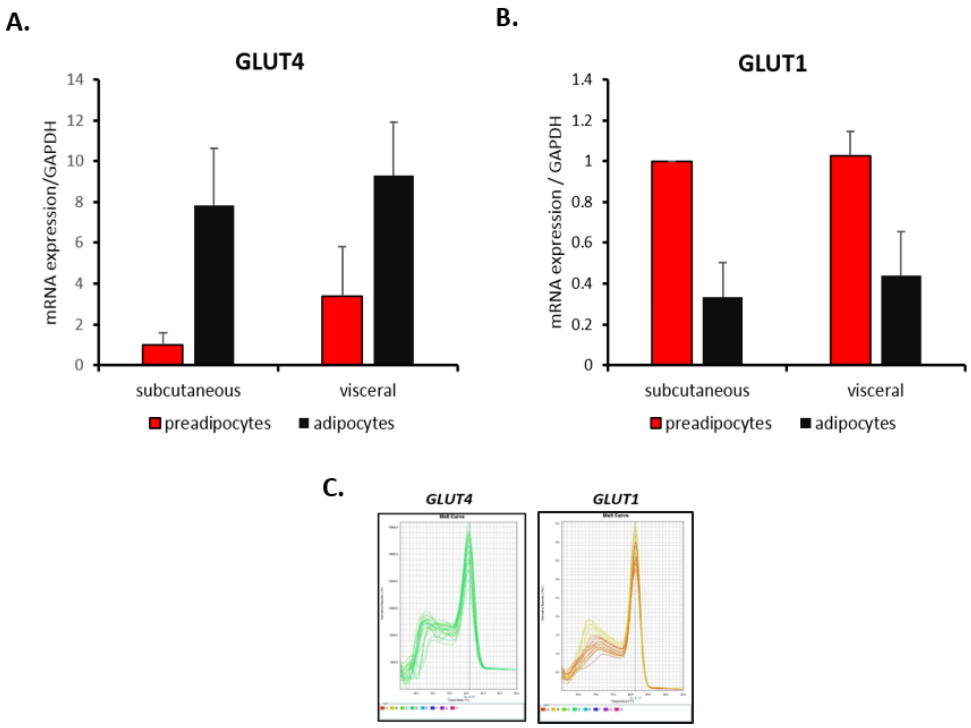


Figure 3-12 mRNA expression of glucose transporters 1 and 4 with differentiation.

Preadipocytes and differentiated adipocytes (day14) from subcutaneous and visceral fat from pre-pubertal children (who were serum starved for 24 hours) in 5mM serum-free media followed by RNA extraction and quantified using SYBR green-based qPCR (A) mRNA expression of GLUT4 (B) GLUT1 (C) Melt curve of GLUT4, GLUT1. GAPDH was used as a house keeping gene.

3.4.7 Basal glucose uptake by human subcutaneous and visceral preadipocytes

To observe whether a difference in glucose transporters reflected changes in glucose uptake ability, we assessed basal 2-deoxy-[^{3}H] d-glucose uptake of preadipocytes and differentiated adipocytes. Preadipocytes were cultured for 24 hours in normal glucose growth media and then changed to normal (5mM/L) or high glucose (25mM/L)-

containing serum-free media for an additional 24 hours and then a non-insulin stimulated glucose uptake assay was performed.

Subcutaneous and visceral preadipocytes have similar basal glucose uptake (3641.3 disintegrations per minute (DPM) vs 4261.33 DPM) when cultured in normal glucose. In high glucose (25mM/L) there was a minor reduction in the uptake levels of both fat depots (3358 DPM vs. 2928.5 DPM), respectively, in comparison to normal glucose (Figure 3-13).

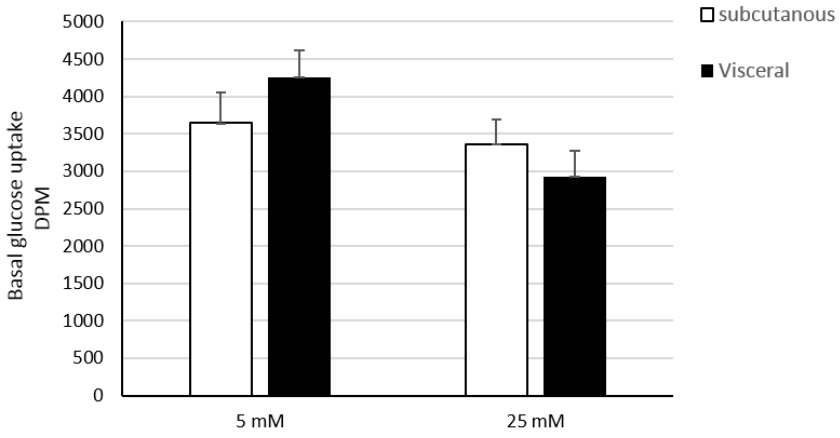


Figure 3-13 Basal glucose transport in human subcutaneous and visceral preadipocytes.

Non-insulin stimulated 2-deoxy-[^{3}H] d-glucose uptake transport was measured in subcutaneous and visceral preadipocytes in two different glucose conditions – 5mM and 25mM. The data represent the basal glucose uptake ± SEM (N=2).

With differentiation, the level of basal glucose uptake was reduced in comparison to preadipocytes for visceral and subcutaneous adipocytes, respectively (3.99 and 3.81fold reduction), and this was expected because glucose uptake by adipocytes is more insulin dependent. Visceral adipocytes also had a significant reduction in glucose uptake ($P<0.05$) when cultured in high glucose media (Figure 3-14).

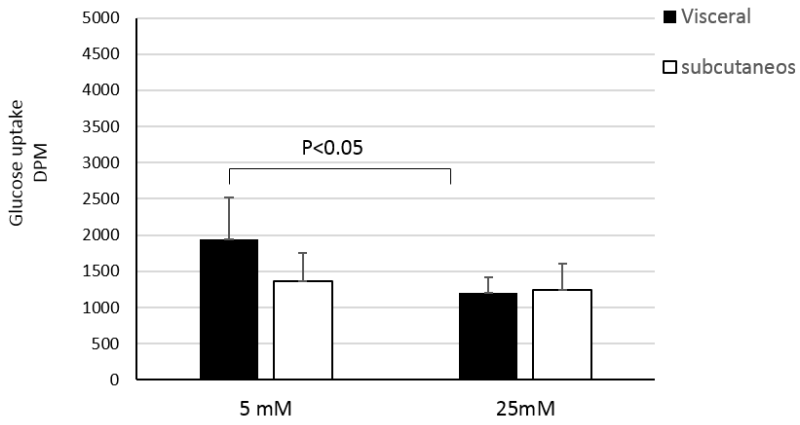


Figure 3-14 Basal glucose transport in human subcutaneous and visceral adipocytes.

Non-insulin stimulated 2-deoxy-[^{3}H] d-glucose uptake transport was measured in subcutaneous (open bars) and visceral (closed bars) adipocytes in high glucose (25mM) and low glucose (5mM). The data represent the basal glucose uptake mean ± SEM (N=2).

To test for adipocyte functionality, insulin-stimulated radioactive glucose uptake was performed for subcutaneous and visceral adipocytes. As predicted, glucose uptake for both subcutaneous and visceral adipocytes was increased after insulin treatment; subcutaneous glucose uptake in comparison to control was (3197vs.1985; P<0.001) and visceral glucose uptake was (3513vs.2194; P<0.001). There was a significant increase in visceral glucose uptake in comparison to subcutaneous glucose uptake (P<0.05) (Figure 3-15).

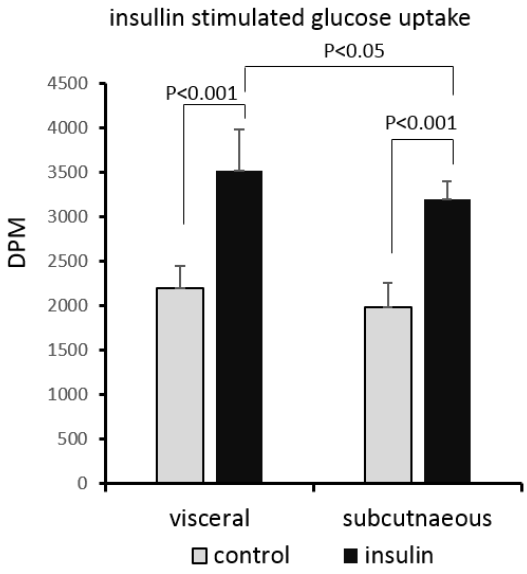


Figure 3-15 Insulin stimulate 2-deoxy-[^{3}H] d-glucose uptake in subcutaneous and visceral adipocytes.

Subcutaneous and visceral adipocytes were serum starved in 5mM glucose-containing serum free media for 24 hours followed by the presence or absence of insulin (100 nM) for 15 minutes. Data is presented as mean ± SEM (N=2).

3.4.8 Characterization of insulin and insulin-like growth factor receptors in subcutaneous and visceral preadipocytes

In 3T3-L1 cell lines, there was a clear shift in IGF-IR and IR receptor distribution with differentiation; preadipocytes expressed higher IGF-IR in comparison to IR (41.9 Vs 1; P<0.001). When the cells differentiate the insulin, receptor is predominant (3.9 Vs.48.5; P<0.001) and levels of the IGF-IR are negligible (Figure 3-16).

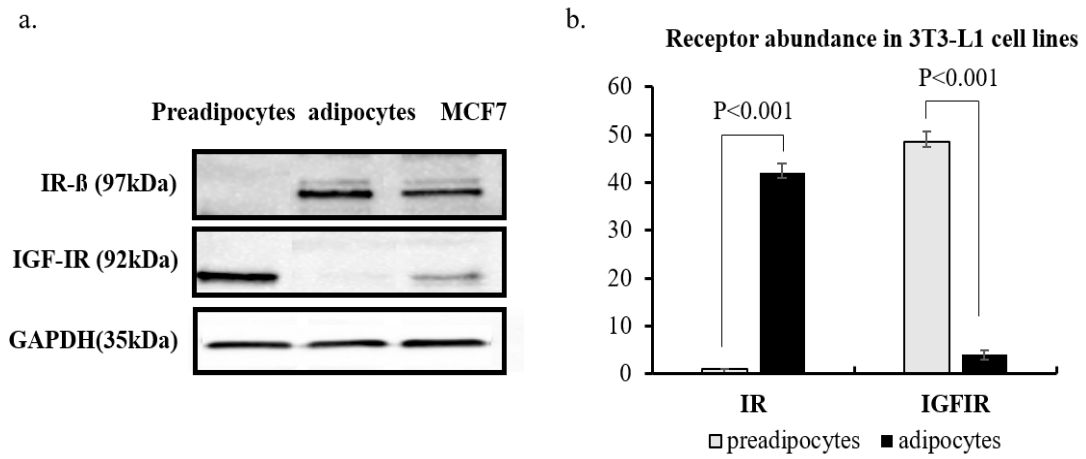


Figure 3-16 Insulin receptor (IR) and insulin-like growth factor I receptor (IGF-IR) abundance in 3T3-L1 cell line in preadipocytes and adipocytes.

(a) Western blot of 3T3-L1 pre-adipocyte cells and differentiated adipocytes (day 7) whole cell lysate extract, showing IR and IGF-IR protein abundance. MCF7 breast cancer cells were used as a positive control and GAPDH worked as loading control. (b) Quantitative densitometry analysis; the data are expressed as mean ± SEM from two experimental repeats and $P<0.05$ was considered as significant.

In human cells, we found that the expression of the IGF-IR receptor decreased with differentiation for subcutaneous ($P<0.01$) and visceral preadipocytes ($P<0.05$). Interestingly, the IGF-IR was significantly higher in subcutaneous preadipocytes in comparison to visceral preadipocytes ($P<0.05$) (Figure 3-17). We then examined protein abundance of the IGF-IR (Figure 3-18) before and after differentiation in subcutaneous and visceral cultures, and a similar pattern was seen; however, subcutaneous preadipocyte predominance in comparison to visceral preadipocytes did not reach statistical significance ($P= 0.082$).

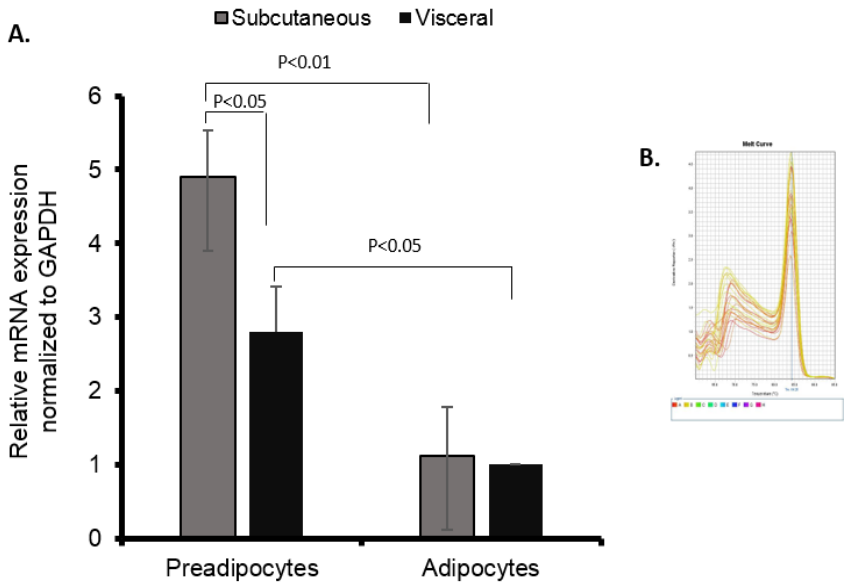


Figure 3-17 mRNA expression of insulin-like growth factor I receptor (IGF-IR) with differentiation in subcutaneous and visceral cultures.

A.IGF-IR mRNA expression was quantified using SYBR green-based qPCR indicating a reduction of expression with differentiation. B.IGF-IR melting curve. Data are expressed as mean ± SEM of duplicate runs (N=3). GAPDH was used as reference gene.

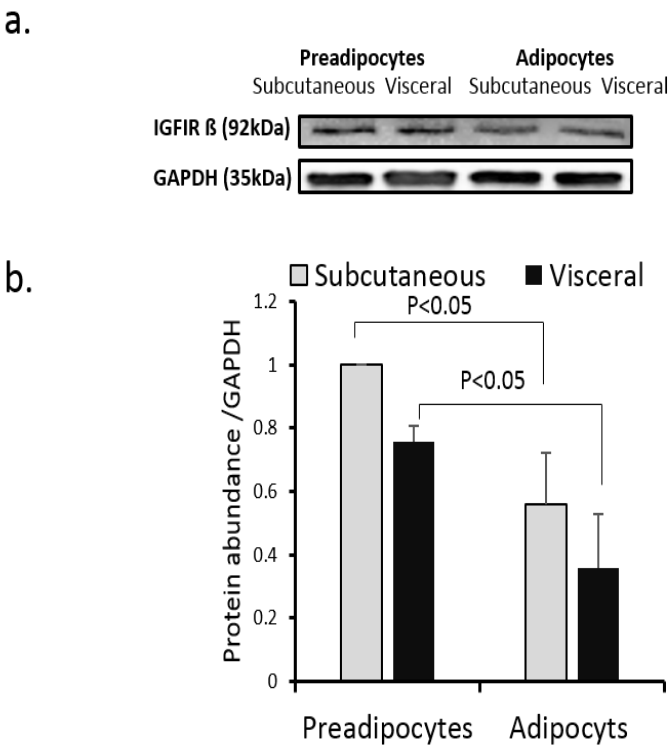


Figure 3-18 Protein abundance of IGF-IR with differentiation in subcutaneous and visceral cultures.

(a) Western blot of IGF-IR indicating decrease abundance with differentiation (b) Semi-quantitative analysis of the western blot (a) Normalized to GAPDH reference protein. Data are expressed as mean ± SEM of three experimental repeats in triplicate (N=3).

Insulin receptor mRNA expression seems to increase with differentiation (Figure 3-19) for subcutaneous and visceral adipocytes, as cells become more insulin dependent. The mRNA expression of total insulin receptor was higher in visceral preadipocytes ($P<0.05$) and adipocytes ($P<0.01$) in comparison to subcutaneous cells.

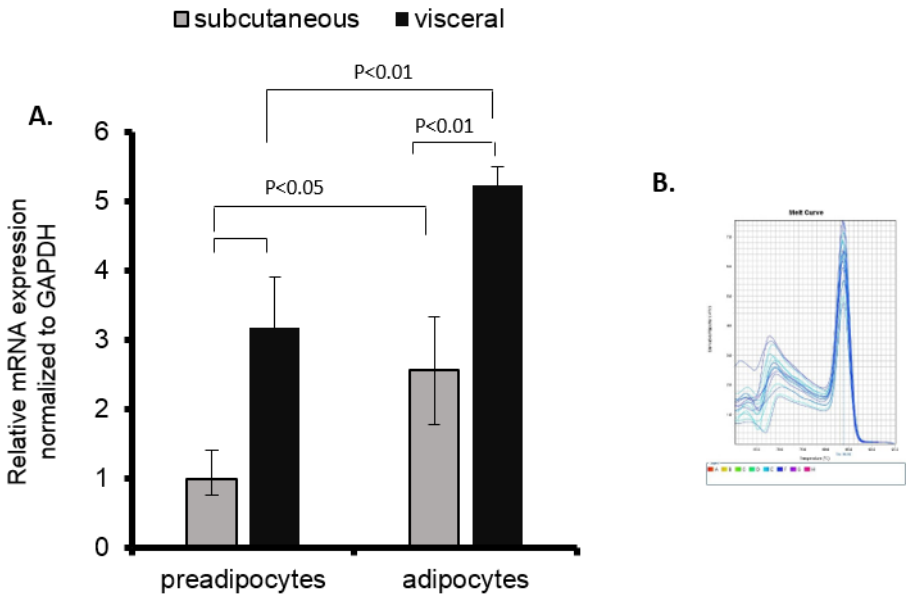


Figure 3-19 Genetic expression of insulin receptor (IR) with differentiation in subcutaneous and visceral cultures.

A. Relative mRNA expression was quantified using SYBR green-based qPCR indicating an increase of expression with differentiation. B. Insulin receptor qPCR melting curve. Data are expressed as mean ± SEM of duplicate runs (N=3). GAPDH was used as reference gene.

3.4.9 Insulin receptor isoform expression with differentiation in subcutaneous and visceral tissue cultures

The insulin receptor has two isoforms due to the alternative splicing of exon 11. IR-B which has exon 11 and IR-A which lacks this exon. A main functional difference between the two isoforms is their affinity for IGF-II. In the previous results section (3.4.8), we indicated that preadipocytes express both the IR and the IGF-IR, and the IR is expressed more with differentiation together with a reduction in the IGF-IR. We examined the distribution of IR-A and IR-B in both preadipocytes and adipocytes. In preadipocytes, visceral preadipocytes have a higher ratio of insulin receptor isoform A in comparison to insulin receptor isoform B (28.2 Vs. 1.5; $P<0.001$) and subcutaneous preadipocytes have a higher ratio of IR-B compared with IR-A (8.9 Vs. 1.0: $P<0.05$). Looking further into the difference between subcutaneous and visceral fat depots, there was a significant higher expression of IR-A in visceral preadipocytes, with a more than 30-fold ($P<0.001$) higher expression in comparison to subcutaneous preadipocytes (Figure 3-20).

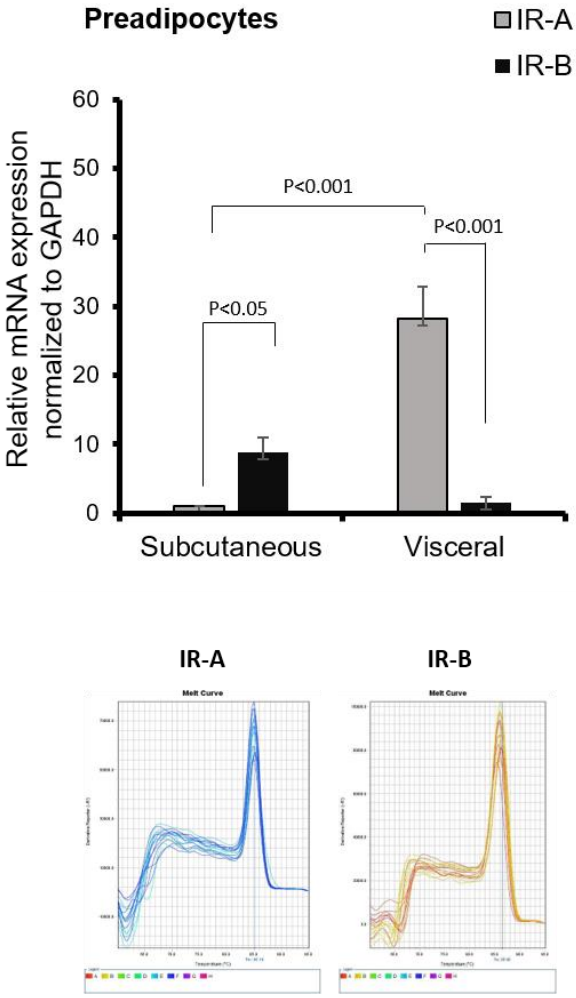


Figure 3-20 Relative genetic mRNA expression of insulin receptor isoforms in preadipocytes from prepubertal children.

Subcutaneous and visceral preadipocytes were serum starved for 24 hours in 5mM/l serum free media followed by RNA extraction and quantified using SYBR green-based qPCR. Data are expressed as mean ± SEM of experiments run in duplicate (N=4). GAPDH was used as reference gene.

In terms of differentiation, the mRNA expression ratio of IR-B significantly increased compared to that of IR-A in subcutaneous and visceral adipocytes (22.4 Vs. 1.0: $P<0.05$) and (45.6 Vs. 33.7; $P<0.05$), respectively. This ratio is a feature of insulin-sensitive tissues. However, visceral adipocytes still maintained a higher ratio of IR-A in comparison to subcutaneous adipocytes ($P<0.01$) (Figure 3-21).

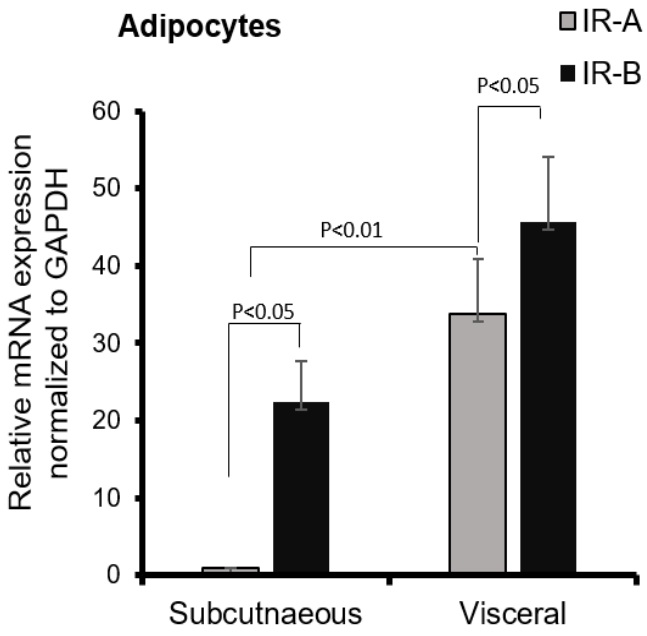


Figure 3-21 mRNA expression of insulin receptor isoforms in differentiated adipocytes from prepubertal children.

Subcutaneous and visceral adipocytes on day 14 of differentiation were serum starved for 24 hours in 5mM/L serum-free media followed by RNA extraction and quantified using SYBR green-based qPCR. GAPDH was used as reference gene. Data are expressed as mean ± SEM of experiments run in duplicate (N=4).

3.5 Discussion

This chapter focused on the differences between visceral and subcutaneous preadipocytes derived from prepuberal children in terms of levels and distribution of IGF receptors, uptake of glucose and levels of GLUT 1 and 4 transporters and we also measured tissue production of IGFs.

The importance of using prepubertal adipose tissue is related to the need to understand adipose tissue distribution during childhood, which is a critical time in the development of childhood obesity. The majority of tissue characterisation studies are based on adult samples, which might be influenced by the pubertal surge of growth hormone, insulin-like growth factors and sex-steroids.

We examined the differences in local production of insulin-like growth factors, and consistent with other studies, IGF-II was predominantly secreted in comparison to IGF-I (Hauner, Röhrig et al. 1998, Gude, Frystyk et al. 2012). Our data indicate that this substantial increase in production of IGF-II was from both subcutaneous and visceral cultures. However, there was no marked depot-specific difference in IGF-II secretion when cultures were subjected to hyperglycaemia. It was previously reported that the metabolic environment could influence the secretion of IGFs (Bäck and Arnqvist 2009) and that intra-uterine hyperglycaemia alters IGF-II gene expression (Ding, Wang et al. 2012). Furthermore, IGF-II production with increased secretion from visceral cultures in comparison to subcutaneous has been observed in cultures obtained from middle-aged obese females (Gude, Frystyk et al. 2012) .

We showed that GLUT1 expression decreased with preadipocyte differentiation, whereas GLUT4 increased: a similar pattern of expression was also reported in cultures obtained from young females (Hauner, Röhrig et al. 1998); however, in older

women a similar increase in GLUT4 was reported with no change in GLUT1 (Perrini, Laviola et al. 2008, Bäck and Arnqvist 2009). Our results indicate that glucose transporter 1 which is an insulin independent transporter was more abundant in preadipocytes in comparison to adipocytes this being reflected in elevated basal levels of glucose uptake (Ebeling, Koistinen et al. 1998), in preadipocytes in comparison to adipocytes. However with differentiation, adipocytes became more insulin dependent and a significant increase in glucose uptake was seen and these related to associated increases in GLUT4. We also observed increased insulin-stimulated glucose uptake and increased GLUT4 levels in visceral adipocytes, compared to subcutaneous adipocytes, which is consistent with findings reported in children (Grohmann, Stewart et al. 2005) and have also been observed in primary cultures of visceral adipocytes from adults (Lundgren, Burén et al. 2004).

Receptors are an important regulator of physiological functions. In adipose tissue, receptor distribution varies with the status of preadipocyte differentiation, with the IGF-IR being expressed in preadipocytes, as seen in the 3T3L1 cell line (Shimizu, Torti et al. 1986, Smith, Wise et al. 1988), and in cultures obtained from mammals (Nouguès, Reyne et al. 1993, Gerfault, Louveau et al. 1999). However, whether or not IGF-IR receptor expression persists in adipocytes is still controversial. We have shown that the IGF-IR significantly decreases with differentiation; however, it was still detected in mRNA expression and protein abundance but at minimal levels. In cultures obtained from middle-aged adults, the expression of the IGF-IR was also detected (Shimizu, Torti et al. 1986) and this was accompanied by increased levels of the insulin receptor (Kern, Svoboda et al. 1989, Bäck and Arnqvist 2009). In contrast, the IGF-IR was not detected in primary cultures obtained from pigs (Richardson, Hausman et al. 1994). Other studies also indicated an increase of insulin receptor

expression with differentiation (Shimizu, Torti et al. 1986, Bäck and Arnqvist 2009). The fat-depot differences in insulin receptor expression showed an increase in insulin receptor expression being observed in visceral fat in comparison to subcutaneous fat in adult subjects of normal weight (Laviola, Perrini et al. 2006) and in the obese (Lefebvre, Laville et al. 1998). As all our data was obtained from samples taken from normal weight children, it might be interesting in future studies to consider comparing biopsies from lean and obese children. Other adults studies however, have reported no fat-depot differences in insulin receptor expression (Bolinder, Kager et al. 1983).

Insulin receptor isoforms have a tissue-specific manner of expression and this expression also varies between species. This indicates that insulin receptor isoforms may serve different physiological functions. For instance, in pigs IR-A is the dominant isoform in subcutaneous and retroperitoneal fat, whereas IR-B predominates in muscles. However, in rodents, IR-A is more expressed in muscles and IR-B is the dominant isoform in epididymal fat, and both IR-A and IR-B are expressed in retroperitoneal fat (Vienberg, Bouman et al. 2011). In humans, studies investigating insulin receptor isoforms are limited and controversial. Although similar IR-B predominance was reported in insulin-sensitive adipocytes (Moller, Yokota et al. 1989), a conflicting study reported that IR-A is predominant in adipocytes in middle-aged overweight female subcutaneous fat (Bäck and Arnqvist 2009). The literature is extremely limited with respect to children; only one study discussed childhood insulin receptor isoforms, examining perirenal fat from a single, 9-month-old female infant and reporting higher expression of IR-A. Although this is consistent with our results, this study was from one individual only and no mention was made regarding the differentiation status of the cells (Seino and Bell 1989). Our data indicate that in children, IR-A is more predominant in visceral preadipocytes, whereas IR-B is

significantly expressed in subcutaneous fat; however, as the cells differentiate into adipocytes, IR-B becomes more prominent. However, visceral adipocytes still maintained a higher expression of IR-A in comparison to subcutaneous adipocytes. As far as we know, this is the only study that has analyzed fat depot differences in insulin receptor isoforms in children. The role of these isoforms is currently of growing interest. Although the literature indicates that IR-A is more involved in prenatal growth and IR-B is linked to metabolism, recent studies suggest a new metabolic function for IR-A promoting glucose uptake and glycogen synthesis in muscle and murine hepatocytes (Diaz-Castroverde, Baos et al. 2016, Lopez-Pastor, Gomez-Hernandez et al. 2019), and that insulin-like growth factor differences in receptor affinity may play an important role in this context.

3.6 Conclusion

Adipose tissue is uniquely distributed throughout the human body, that allows for biological differences in cellular metabolism and endocrine function between fat depots. In this chapter we examined differences in IGF-IR, insulin and insulin receptor isoforms A and B in subcutaneous and visceral preadipocytes and post differentiation adipocytes. IGF-IR levels were higher in preadipocytes and decreased with differentiation, while the insulin receptor showed an increased expression. In agreement with our hypothesis, insulin receptor isoforms showed a predominance of IR-A in visceral cells in comparison to subcutaneous. As insulin like growth factor II is known to have a higher affinity to insulin receptor isoform A, this encourages us next to examine how IGF-II regulates preadipocyte physiological growth and metabolic functionality in a fat-depot manner.

Chapter 4: The role of Insulin like growth factor II (IGF-II) in preadipocyte regulation

4.1. Introduction

The IGF system has an established role in adipose tissue growth and metabolism. The system consists of insulin-like growth factor I (IGF-I), insulin-like growth factor II (IGF-II), surface receptors (IGF-IR, IR and IGF-IIR/M6P) and six regulatory binding proteins (IGFBPs 1-6). The IGFs promote the growth of many tissues like muscle and bone (Zapf and Froesch 1986) and adipose tissue is considered a major target of IGF hormones. IGF-I is a potent promoter of preadipocyte proliferation and differentiation (Wright and Hausman 1995, Gregoire, Smas et al. 1998) and fat-depot differences in IGF-I responses have been reported (Grohmann, Sabin et al. 2005). IGF-I regulates preadipocytes directly or indirectly by facilitating the actions of growth hormone (GH) on adipose tissue (Berryman, Glad et al. 2013). In addition to adipose tissue growth, IGF-I acts as a metabolic regulator of glucose uptake, lipogenesis and lipolysis (Berneis, Vosmeer et al. 1996).

Dysregulation of IGF binding proteins and ultimately IGF-I bioavailability is seen in obesity and metabolic syndrome (McMillen, MacLaughlin et al. 2008, Saydah, Ballard-Barbash et al. 2009).

IGF-II is recognized for being an embryonic and placental growth factor, but its physiological role postnatally is still to be determined. The reason for this might be because IGF-II is not expressed postnatally in mouse or rat models, whereas humans maintain extremely high levels of IGF-II throughout life with concentrations considerably higher than those of IGF-I (Birnie, Ben-Shlomo et al. 2012).

IGF-II expression has been strongly related to weight and adiposity; the level of IGF-II gene methylation is associated with birth weight (Bennett, Wilson et al. 1983) and the expression of IGF-II *in utero* promotes adipogenesis and fat storage during

pregnancy (Whitaker and Dietz 1998). Additionally, the methylation status of the IGF-II gene at birth has been linked to early childhood weight, (Le Stunff, Fallin et al. 2001) and the level of IGF-II in the circulation during childhood has been closely related to fat mass (Ong, Kratzsch et al. 2002). In adults, polymorphic genetic differences in IGF-II expression correlated with weight gain; homozygous individuals with Apal AA had significantly higher IGF-II levels and this was associated with less body weight and lower risk of pathological body mass index (BMI) in comparison to those with Apal GG who had lower levels of IGF-II (O'Dell, Miller et al. 1997, Gaunt, Cooper et al. 2001). Furthermore, IGF-II levels have been proposed as a prognostic marker to predict future weight gain because lower baseline circulating levels of IGF-II were associated with a higher risk of obesity and future weight gain (Sandhu, Gibson et al. 2003). Despite these strong suggestions of a role for IGF-II in adipose tissue growth and metabolism, a regulatory role is still largely unknown. Using matched pairs of subcutaneous and visceral adipocyte cultures from children we investigated the physiological role of IGF-II in adipose tissue growth, and we speculated a distinct effect on subcutaneous and visceral cell growth, due to differences in the differential distribution of insulin receptor isoforms between the fat depots.

4.2. Aims and objectives

Our aim is to study if IGF-II has a physiological regulatory role on adipose tissue directly effecting preadipocytes number and their ability to differentiate and mature, this will be assessed by:

A) Examine the proliferative effect of IGF-I, IGF-II and insulin on preadipocytes using subcutaneous and visceral human cultures.

B) Study the role of IGF-II in subcutaneous and visceral preadipocyte differentiation using:

1. Oil red O staining triglyceride staining
2. Changes in the genetic expression and protein abundance of differentiation markers

C) Examine the effect of IGF-II on preadipocyte maturation by the assessment of terminal differentiation markers like; insulin receptor, glucose transporter 4 and fatty acid synthase.

D) Examine the acute effects of IGF-II treatment on adipocytes; the effect on receptor expression of IGF-IR, IR, IR isoforms and the subsequent metabolic consequences.

4.3. Materials and methods

4.3.1. 3T3-L1 cell line culture.

3T3-L1 preadipocytes were used for optimization purposes. Preadipocytes were grown in T75 flasks to 70% confluency and then plated into 24 well plates with a seeding density of 0.03 $X10^6$ with proliferation being assessed using a tritiated thymidine incorporation assay as described in section (2.2.4).

4.3.2. Child fat biopsy preparation, culture and differentiation

Paired biopsies from subcutaneous and visceral fat were prepared and cultured as described in the methods section (2.2.5). For differentiation experiments, preadipocytes were differentiated with or without continuous exposure to IGF-II at different concentrations.

4.3.3. Tritiated thymidine incorporation Assay (TTI)

A tritiated thymidine incorporation assay (TTI) was used to monitor proliferation in primary human preadipocytes, Cells were seeded into 24 well plates with a seeding density of 0.017 X 10^6 in growth media and allowed to attach for at least 12 hours. Complete media was replaced with SFM for 24 hours to allow the cells to become quiescent and were dosed the following day. After 48 hours 0.1µCi of [^{3}H] thymidine per well was added for the last 4 hours at 37^{o}C. Media was removed, and cells were incubated with 5% trichloroacetic acid (TCA) at 4^{o}C for 10 minutes. The TCA was removed, and the cells were lysed in 1M sodium hydroxide for 1 hour at room temperature. The incorporation of [^{3}H] thymidine into DNA was determined by scintillation counting; a sample (300µl) was mixed with 2ml of scintillation fluid and counted on a β-Scintillation Counter. Data was recorded as disintegration per minute (DPM). Further description of the equipment and protocol is described in section (2.5).

4.3.4. Trypan blue dye exclusion assay

0.4% Trypan blue dye was used for preadipocyte cell counting; cells were harvested from culture plates and mixed with trypan blue dye in a 1:1 ratio. Cell number was determined using a haemocytometer slide and bright white cells were counted as viable cells. Protocol is described in section (2.2.3.2).

4.3.5. Western immunoblotting

Preadipocyte differentiation markers, glucose transporter 4, insulin receptor protein abundance and fatty acid synthase (FASN) were visualized using SDS-PAGE and western blotting. Protocol and antibody concentrations are described in section (2.4). Quantification of band intensities was determined by Image Lab 1.46r software.

4.3.6. RNA extraction and RNA- cDNA reverse transcription

RNA was extracted using Trizol reagent (Invitrogen) as outlined in section (2.6.2): one µg of RNA was reverse transcribed to cDNA using a high capacity RNA-to-cDNA kit (Applied Biosystems) as described in section (2.6.5).

4.3.7. Quantitative Polymerase Chain Reaction (qPCR)

mRNA expression was assessed using SYBR green-based qPCR. The PCR reaction was analyzed using StepOnePlus and relative mRNA levels were determined using the $2^{-\Delta Ct}$ method after normalization to the GAPDH reference gene. For protocol details and primer sequencing refer to section (2.6.6)

4.3.8. Glucose uptake

[^{3}H]2-deoxyglucose uptake was performed for IGF-II and insulin-stimulated cells: the stimulus was added for 15 minutes and glucose uptake was quantified using a β-Scintillation Counter. Data are presented as disintegrations per minute (DPM). Further protocol details are described in section (2.7)

4.3.9. Oil red O Triglyceride staining

Oil red O (ORO) staining being performed on day 14 post differentiation for human cultures; further details of ORO is described in section (2.3). Quantification of the ORO stain was carried out using spectrophotometry (FLUOstar OPTIMA, BMG LABTECH) at 490nm.

4.3.10. Statistical analysis

One-way ANOVA followed by a least significant difference (LSD) post-hoc test was used for multiple comparisons (subcutaneous and visceral) at day 0 and day 14 post differentiation. The software used for analysis was SPSS 12.0.1 for Windows. A statistically significant difference was considered to be present at $p< 0.05$.

4.4. Results

4.4.1. Optimization of a proliferation assay for the 3T3-L1 cell line using a tritiated thymidine incorporation (TTI) assay

Because children's biopsies are very precious, we first used the 3T3-lL cell line to optimize the assay. The tritiated thymidine incorporation assay is one of the most accurate because it is directly measuring DNA synthesis (Madhavan 2007). 3T3-L1 cells were treated with IGF-I (0-500 ng/ml) (Boney, Smith et al. 1998). IGF-I induced a significant increase in proliferation from 31.25 ng/ml in comparison to control with a peak response observed at 125 ng/ml ; the fold change increase was respectively; (31.25 ng/ml 2.5; $p<0.01$), (62.5ng/ml 3.3, $p<0.001$), (125 ng/ml;4.8; $p<0.01$), (250 ng/ml; 2.8; $p<0.01$), (500 ng/ml;2.4; $p<0.05$). 7.8 and 15.62ng/ml IGF-I were too low to elicit a significant response (Figure 4-1).

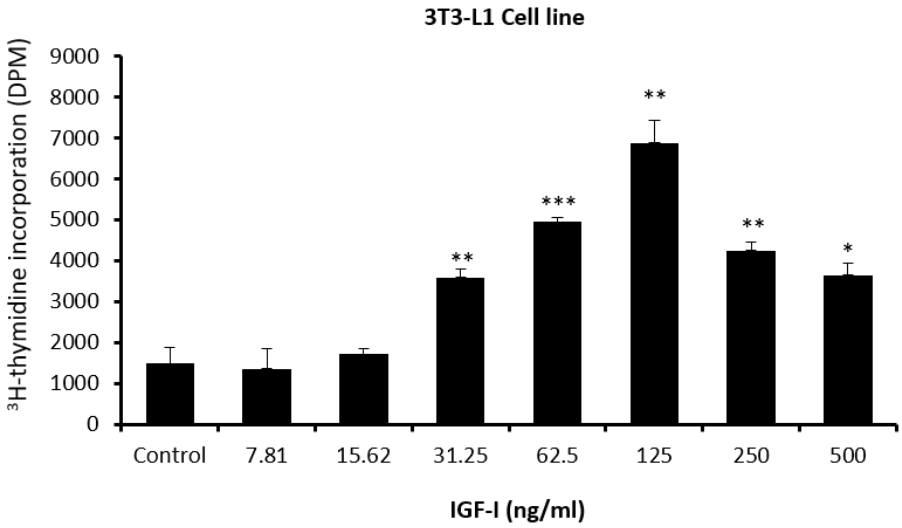


Figure 4-1 Assessment of proliferation in 3T3-L1 cells using a tritiated thymidine incorporation assay (TTI) following IGF-I treatment

3T3-L1 preadipocytes were seeded in 24 well plates at a density 0.03×10^6 per well. Following cell attachment, preadipocytes were serum starved for 24 hours and then dosed with IGF-I(0-500ng/ml) for 48 hours. Cell proliferation was detected using a TTI assay. The graph

represents the mean ±SEM of three independent repeats each performed in duplicate (* P<0.05, ** P<0.01, *** P<0.001).

To confirm the results obtained using the TTI assay, the effects of IGF-I on 3T3-L1 cell growth was also assessed by cell counting using a trypan blue dye exclusion assay (Figure 4-2). A similar pattern of proliferation was seen with a peak in cell growth observed at 125 ng/ml.

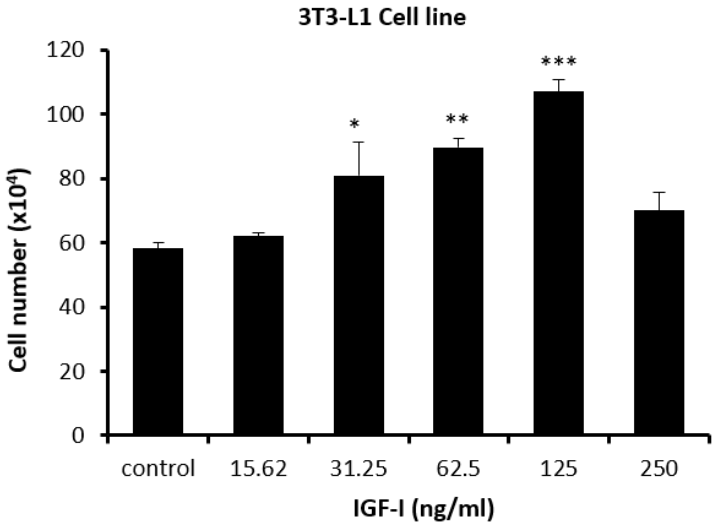


Figure 4-2 Assessment of 3T3-L1 proliferation following IGF-I treatment using trypan blue dye exclusion assay.

Preadipocytes were serum starved in 5mM glucose serum free media (24h) followed by IGF-I treatment (0-500 ng/ml). Trypan blue cell counting was performed after 48 hours of treatment where bright cells were considered viable. The graph represents the mean ±SEM of two independent repeats each performed in duplicate (* p<0.05, ** p<0.01, *** p<0.001).

IGF-II was less effective in inducing proliferation in 3T3-L1 cells than IGF-I: this probably being due to its lesser affinity to the IGF-IR. 62.5 ng/ml elicited a 3.4 fold increase (p<0.01), 125 ng/ml a 2.8 fold increase (p<0.05) with the highest response

observed with 250 ng/ml is 3.6 fold increase in comparison to control ($p<0.01$). The other doses did not induce an effective proliferative response (Figure 4-3).

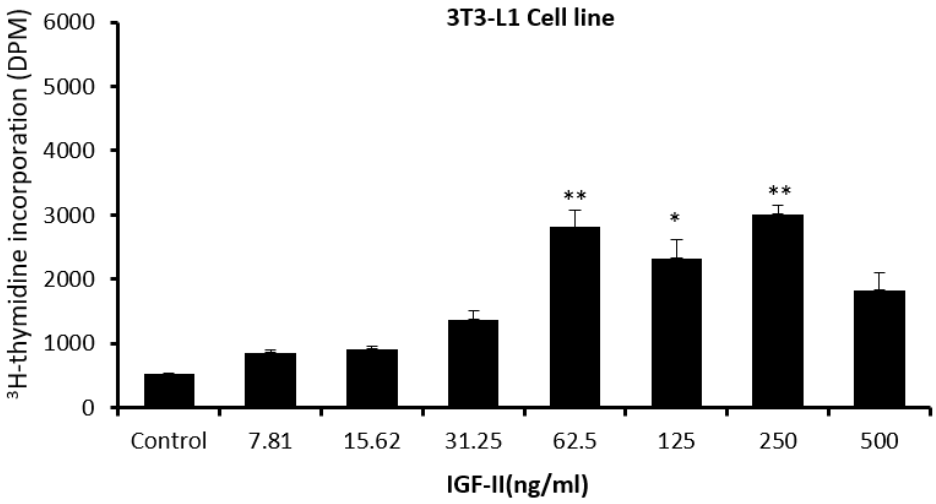


Figure 4-3 Assessment of proliferation in 3T3-L1 cells using a tritiated thymidine incorporation assay (TTI) following IGF-II treatment.

3T3-L1 preadipocytes were seeded in 24 well plates at a density 0.03×10^6 cells per well, followed by serum starvation for 24 hours. They were the dosed with IGF-II (0- 500ng/ml) for 48 hours. TTI assay was used to measure preadipocyte proliferation. The graph represents the mean ±SEM of three independent repeats each conducted in duplicate (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

4.4.2. The effect of IGF-I on human subcutaneous and visceral preadipocyte proliferation.

After optimization of the tritiated thymidine incorporation (TTI) assay using 3T3-L1 cells, we investigated fat-depot differences in proliferation using human cultures. Paired subcutaneous and visceral preadipocytes were serum starved in normal glucose (5mM) serum free media for 24 hours followed by IGF-I treatment (0-500 ng/ml) for 48 hours. IGF-I was more potent in stimulating growth in subcutaneous compared

with visceral preadipocytes. In comparison to control there was a (1.4; $p<0.05$), (1.7; $p<0.05$), (2.1; $p<0.001$), (1.8; $p<0.05$), (1.6; $p<0.05$) and (1.5; $p<0.01$) fold increase in proliferation for the following IGF-I doses 7.8, 15.6, 31.2, 62.5 ,125, 250 respectively (Figure 4-4 A). Visceral preadipocytes were less responsive to IGF-I than subcutaneous at the higher doses and the fold of increase in comparison to control was 1.4 ($p<0.05$) and 1.9 ($p<0.001$) for the doses 15.6 and 31.2 ng/ml with a diminution of proliferative responses with other doses (Figure 4-4 B). The IGF-I responses showed a bell-shaped curve, representative of a typical biological response.

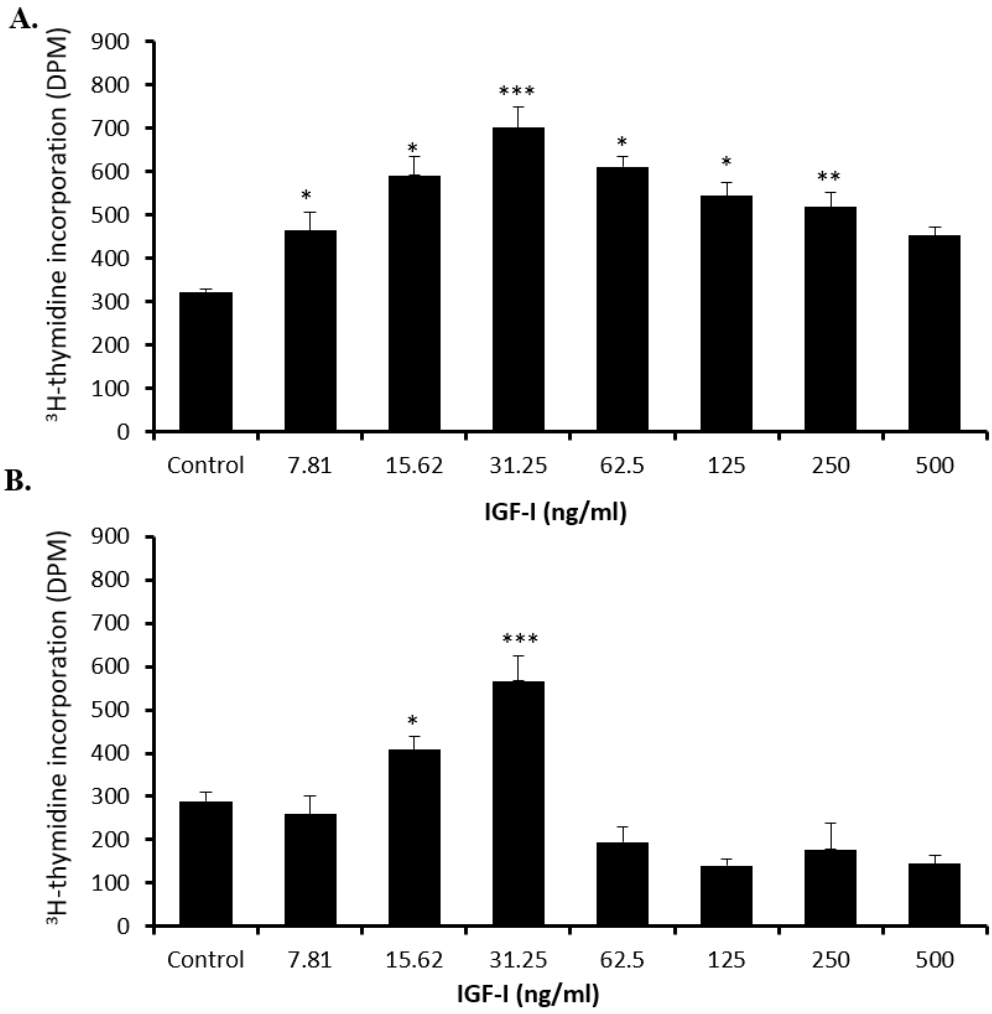


Figure 4-4 Effect of IGF-I treatment on subcutaneous and visceral preadipocyte proliferation.

Tritiated thymidine incorporation assay (TTI) was used to assess proliferation in. subcutaneous (A) and visceral (B) preadipocytes. Cells were seeded at $0.017x10^6$ cells/well and treated with IGF-I (0 to 500 ng/ml) in 5mM glucose SFM. TTI was performed after 48h of treatment. The graph represents the mean ±SEM of three independent repeats each performed in duplicate (n=3).
(* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

4.4.3. Effect of IGF-II on human subcutaneous and visceral preadipocyte proliferation.

A role for IGF-I in preadipocyte growth regulation is well established but less is known about the role of IGF-II. We investigated the effect of IGF-II treatment (0-500 ng/ml) on subcutaneous and visceral preadipocytes proliferation using a TTI assay.

IGF-II treatment induced subcutaneous preadipocytes proliferation in comparison to control with 15.6 ng/ml treatment (1.2 fold; $p<0.05$), 31.25 ng/ml (1.4 fold; $p<0.05$) and 62.5 ng/ml (1.5 fold; $p<0.01$) (figure 4-5 A). Interestingly, IGF-II elicited a greater proliferative effect in visceral preadipocytes in comparison to subcutaneous as the fold change increase in comparison to control was (1.3 fold; $p<0.05$), (1.7 fold; $p<0.05$), (1.8 fold; $p<0.05$), (2.0 fold; $p<0.001$) for the doses 7.8 ,15.6, 31.2, 62.5 ng/ml respectively. The higher doses of IGF-II; 125 ng/ml induced lesser proliferative response (Figure 4-4 B).

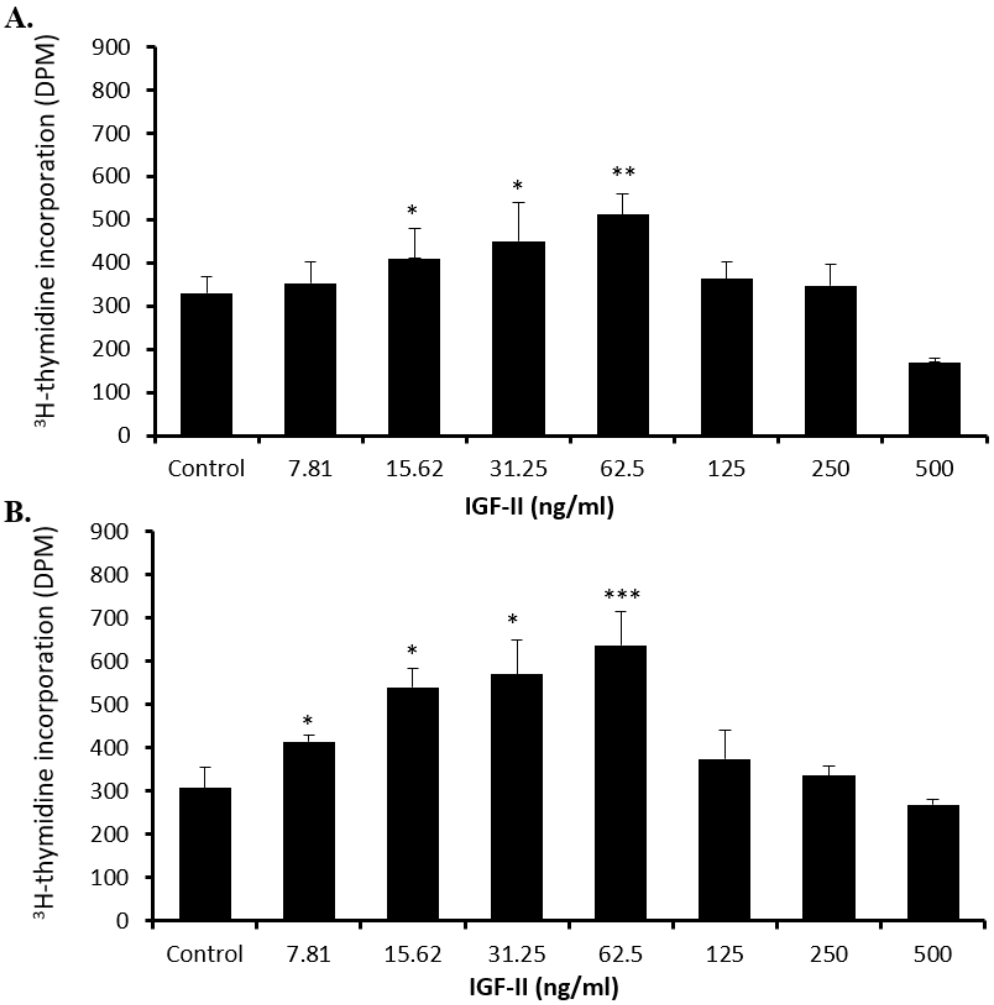


Figure 4-5 Proliferative effect of IGF-II on human subcutaneous and visceral preadipocytes.

The proliferation **of A.** Subcutaneous and **B.** visceral preadipocytes was determined using a TTI assay. Cells were seeded at $0.017x10^6$ cells and were treated with multiple concentrations of IGF-II (0ng/ml to 500 ng/ml) in (5mM) glucose SFM. TTI was performed after 48hrs of treatment. The graph represents the mean±SEM of three independent repeats each performed in duplicate (n=3). (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

4.4.4. Effect of insulin on human subcutaneous and visceral preadipocytes on proliferation.

In comparison to IGFs, insulin has minimal effects on proliferation of subcutaneous or visceral preadipocytes. Preadipocytes were treated with insulin (0-500 ng/ml) and no significant increase in proliferation was detected in either fat depot. The highest fold change increase in comparison to control was 1.1 in subcutaneous preadipocytes and 1.05 in visceral preadipocytes (figure 4-6).

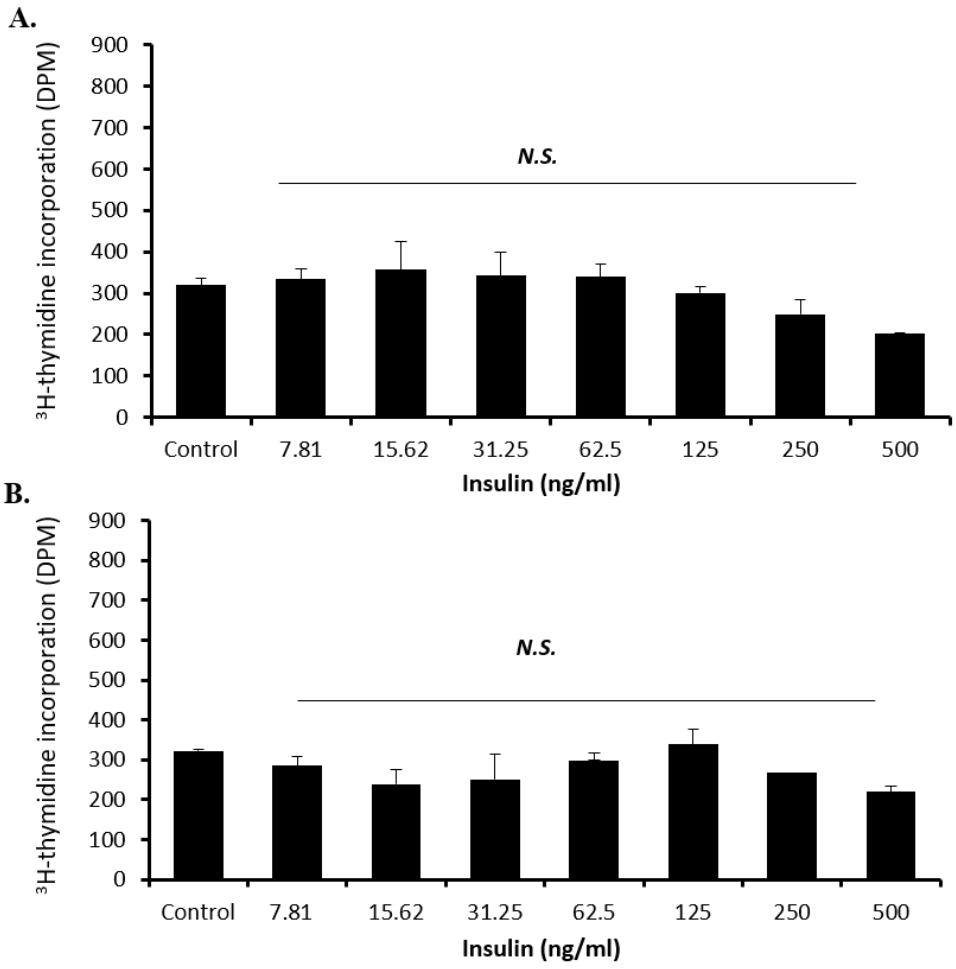


Figure 4-6 Effect of insulin treatment on human subcutaneous and visceral preadipocyte proliferation.

A. Subcutaneous preadipocytes and **B.** visceral preadipocytes were seeded at 0.017x10^{6}cells and treated with different concentrations of insulin (0- 500 ng/ml) in 5mM glucose SFM. Proliferation was determined using a TTI assay after 48h. The

graph represents the mean ±SEM of three independent repeats each performed in duplicate (n=3). Statistical significance was determined at ($p<0.05$).

4.4.5. Effect of IGF-II treatment on human subcutaneous and visceral preadipocyte differentiation.

Adipose tissue growth consists of an increase in cell number as well as size. After examining the effect of IGF-II on preadipocyte proliferation, we investigated the role of IGF-II in preadipocyte differentiation in a depot-specific manner. As illustrated previously in section (4.4.3), IGF-II is more effective at doses less than 100 ng/ml: for that reason, we chose the lowest dose that caused a significant proliferative effect (7.5 ng/ml) and the dose with maximal effect on proliferation (62.5 ng/ml) to examine the role of IGF-II in preadipocyte differentiation (these doses are within the physiological concentration) (Philpott, Sanders et al. 1994). Visceral and subcutaneous preadipocytes were differentiated for 14 days with normal glucose (5mM/L) differentiation media or differentiation media supplemented with the stipulated concentrations of IGF-II concentrations.

4.4.5.1. *Assessment of IGF-II on preadipocyte differentiation using Oil red O triglyceride staining.*

Oil red O staining was performed on day 14 post differentiation. Spectrometry quantification of absorbance of the Oil red O indicated that IGF-II (62.5 ng/ml) enhanced fat deposition in subcutaneous fat in comparison to control; this was observed under the microscope (Figure 4-7 A) and by Oil red O staining analysis, as the absorbance quantification showed a significant increase in comparison to control (0.847 Vs. 0.3; $p<0.01$) (figure 4-7 B). In terms of visceral preadipocyte differentiation, interestingly IGF-II reduced the amount of preadipocyte

differentiation as seen under the microscope (Figure 4-7 A). Oil red O absorbance analysis showed a reduction in triglycerides with IGF-II treatment (62.5 ng/ml) in comparison to control (0.3 Vs. 0.5; P<0.05) (Figure 4-7 B)

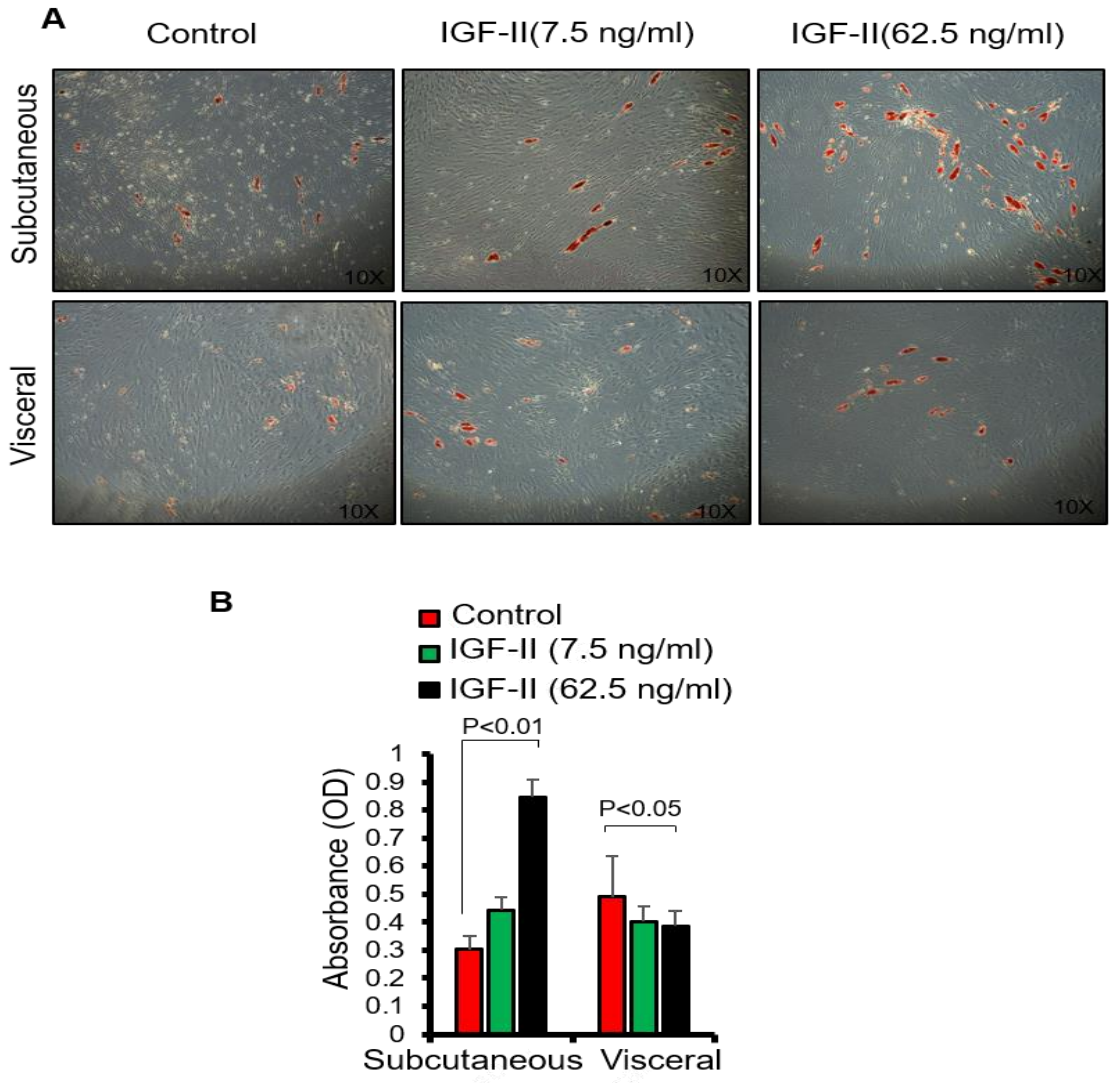


Figure 4-7 Effect of IGF-II treatment on subcutaneous and visceral preadipocyte differentiation.

Subcutaneous and visceral preadipocytes were differentiated for 14 days in the presence or absence of IGF-II at (7.5 ng/ml) or (62.5 ng/ml). (A) micrograph of differentiated preadipocytes stained with Oil red O at day 14 of differentiation. Magnification at (X10). (B) Quantitative Oil red O staining absorbance analysis of (A) showing a reduction in Oil red O absorbance in visceral adipocytes ($p<0.05$) and an increase in the absorbance in subcutaneous differentiated adipocytes ($p<0.01$) with IGF-II treatment (62.5 ng/ml). The data are expressed

as the mean ± SEM of duplicate runs from three individual biopsies (n=3). Statistical analysis was performed using one-way ANOVA on day 14 of differentiation.

4.4.5.2. Assessment of IGF-II on markers of differentiation in subcutaneous and visceral cultures.

To further confirm the effect of IGF-II on preadipocyte differentiation, subcutaneous and visceral preadipocytes were differentiated for 14 days with normal glucose differentiation media (control) or differentiation media supplemented with the IGF-II (7.5 ng/ml, 62.5 ng/ml). Whole cell lysate was extracted on day 14 post differentiation for western blotting analysis for assessment of peroxisome proliferator-activated receptor gamma (PPARγ) and adiponectin which are known to be increased with adipogenesis, as we previously showed in section (3.4.3.3) and illustrated in figure (4-8). The protein abundance of the differentiation markers PPARγ and adiponectin showed a significant increase in relative fold-change in comparison to control (1.86 vs.1.0 for adiponectin; $p<0.05$) and (3.8 vs. 1.0 for PPARγ; $p<0.01$) in subcutaneous adipocytes when treated with 62.5 ng/ml IGF-II. This IGF-II induced increase in PPARγ was mainly due to an increase in PPARγ2. In contrast, a decrease in the relative fold-change of protein abundance of the differentiation markers in comparison to control was observed for adiponectin (0.4 vs. 0.9; $P<0.05$) and PPARγ (0.3 vs. 1.2; $P<0.05$) for visceral adipocytes when treated with the same dose of IGF-II. IGF-II at 7.5ng/ml was not sufficient to induce significant changes. The changes are illustrated in (Figure 4-9).

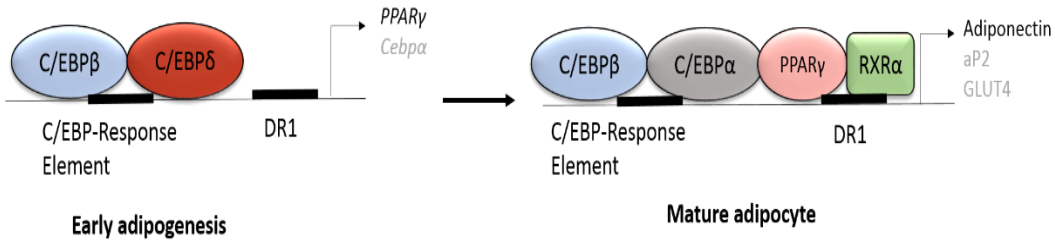


Figure 4-8 Illustration of preadipocyte early and late differentiation markers. *The figure is adapted from (Ràfols 2014).*

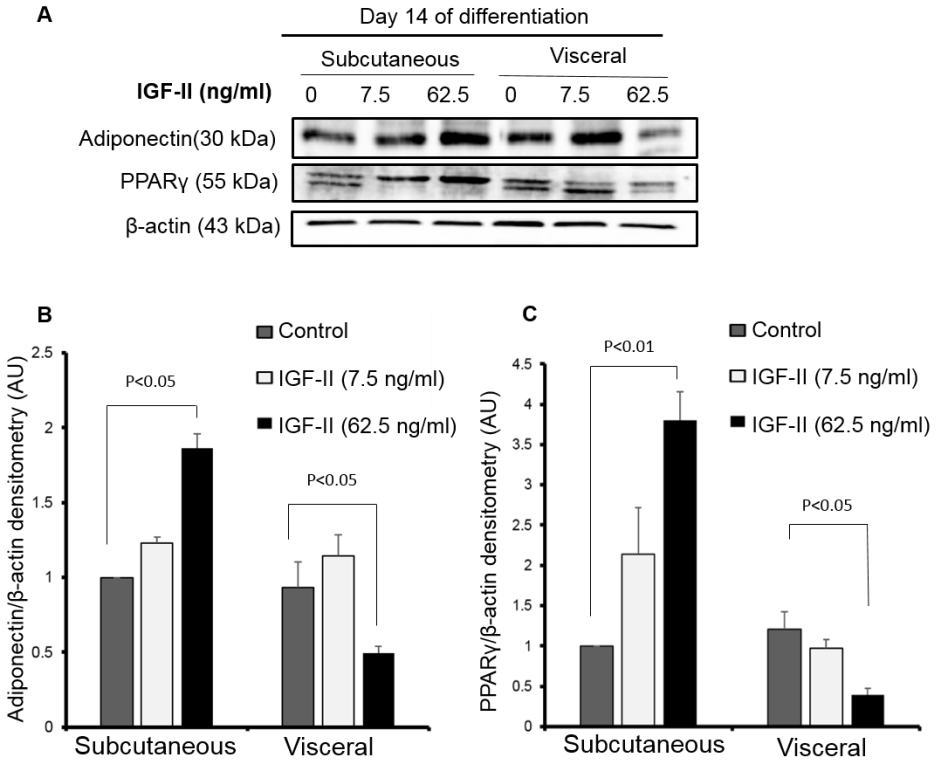


Figure 4-9 IGF-II promoted differentiation of subcutaneous but not visceral preadipocytes.

Subcutaneous and visceral preadipocytes were differentiated for 14 days in the presence or absence of IGF-II at (7.5 ng/ml) or (62.5 ng/ml). (A) Western immunoblotting of the differentiation markers PPARγ and adiponectin. (B) Densitometry of the western blot showing protein abundance of adiponectin after normalization to the reference protein β-actin. (C) Densitometry quantification of the differentiation marker PPARγ after normalization to β-actin. The data are expressed as the mean ± SEM of and each western blot densitometry is

representative of experiments performed in triplicate from three individual biopsies (n=3). Statistical analysis was performed using one-way ANOVA on day 14 of differentiation.

4.4.6. Effect of differentiation with IGF-II on fat metabolism in subcutaneous and visceral cultures.

Insulin receptor signaling is known to activate the genetic transcription of fatty acid synthase (FASN), lipoprotein lipase (LPL) and glucose transporter 4 (GLUT4). Insulin will also cause GLUT4 translocation to the cell membrane that induces glucose uptake to the fat cell (Czech, Tencerova et al. 2013). In addition, these genes are also known to increase with differentiation (Moseti, Regassa et al. 2016). To further confirm that IGF-II reduced differentiation and fat deposition in visceral preadipocytes whilst enhancing it in subcutaneous preadipocytes, we examined the protein abundance of the insulin receptor, GLUT4 and FASN using western blotting after 14 days of differentiation of paired subcutaneous and visceral preadipocytes with IGF-II at two doses (7.5 ng/ml and 65.5 ng/ml).

4.4.6.1. Effect of IGF-II on insulin receptor abundance with differentiation.

IGF-II enhanced insulin receptor protein abundance in subcutaneous adipocytes and the relative fold change in comparison to control was 2.2 vs.1.0 fold ($p<0.001$) for 7.5ng/ml IGF-II and 3.1 vs.1.0 fold ($p<0.001$) for 62.5 ng/ml IGF-II. In contrast, with visceral adipocytes there was an IGF-II-induced, significant reduction in insulin receptor abundance in comparison to control; the relative fold change reduction was significant for both doses 7.5 ng/ml (1.0vs. 1.4; $P<0.05$) and 62.5 ng/ml (0.9vs.1.4; $P<0.01$). When comparing fat depots, there was a significant difference in insulin receptor protein abundance with IGF-II (62.5 ng/ml) increasing insulin receptor

abundance 2.2-fold in subcutaneous adipocytes in comparison to visceral (P<0.001) (Figure 4-10).

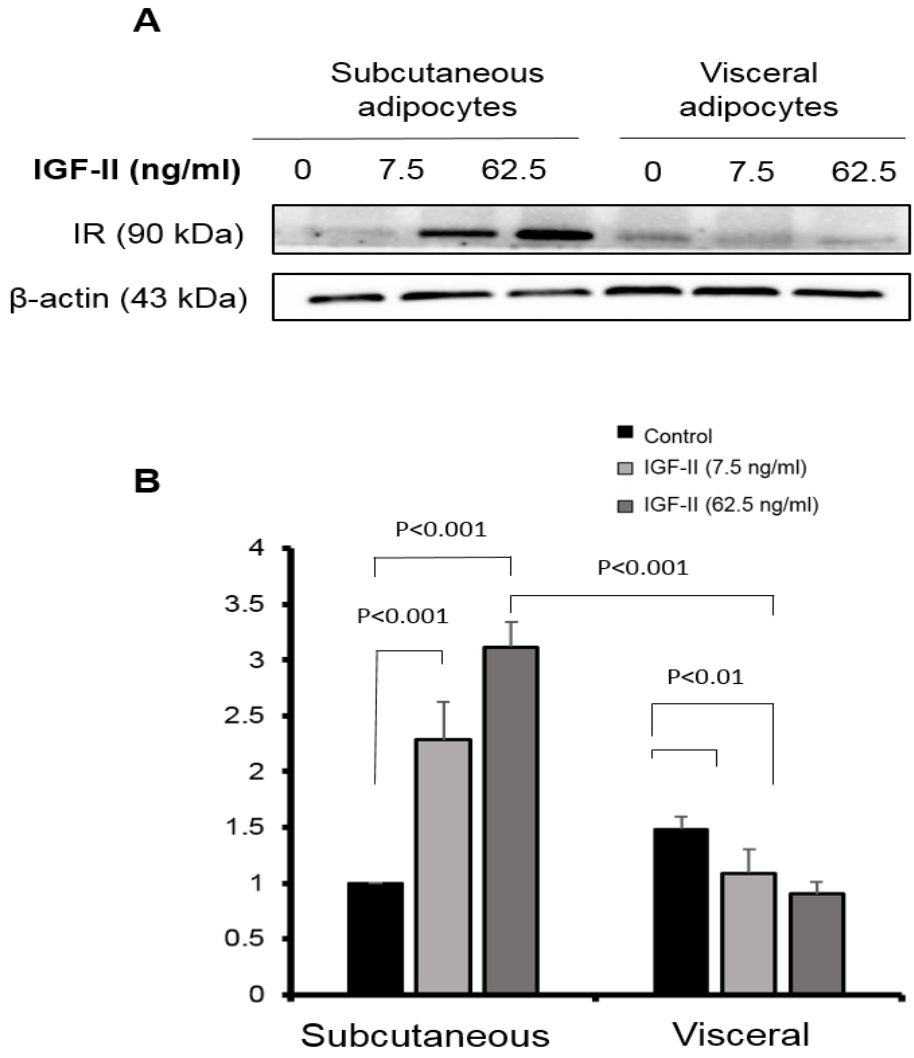


Figure 4-10 Effect of IGF-II on insulin receptor protein abundance with differentiation.

(A)Western blotting of paired subcutaneous and visceral preadipocytes differentiated for 14 days with IGF-II (7.5 ng/ml or 62.5 ng/ml) showing insulin receptor protein abundance. (B) Densitometry western blot analysis of (A). β-actin was used to ensure equal loading of samples. Densitometry represents the mean ± SEM of three experimental repeats from three individual biopsies (n=3). Statistical analysis was performed using one-way ANOVA on day 14 of differentiation.

4.4.6.2. Effect of IGF-II treatment on the abundance of the glucose transporter 4 (GLUT4) and consequent glucose uptake with differentiation in subcutaneous and visceral cultures.

A consistent decrease in GLUT4 protein abundance was seen in visceral adipocytes when differentiated with IGF-II at 7.5 ng/ml (0.8vs.1.4; $p<0.01$) and at 62.5 ng/ml (0.7vs. 1.4; $p<0.01$) (Figure 4-11). This was also reflected by a 16% reduction in insulin-stimulated radioactive 2DG glucose uptake by visceral adipocytes in comparison to control ($p<0.01$) (Figure 4-12) following IGF-II (62.5ng/ml) treatment. GLUT4 relative protein abundance increased in subcutaneous adipocytes with IGF-II (62.5 ng/ml) (1.5vs.1.0; $P<0.05$) in comparison to control, However, the increase in abundance of GLUT4 at 7.5 ng/ml IGF-II was insignificant (Figure 4-11). Insulin-stimulated glucose uptake was also significantly increased in subcutaneous adipocytes using the higher dose of IGF-II in comparison to control (2019 DPM vs.1678 DPM; $P<0.01$) as seen in figure (4-12).

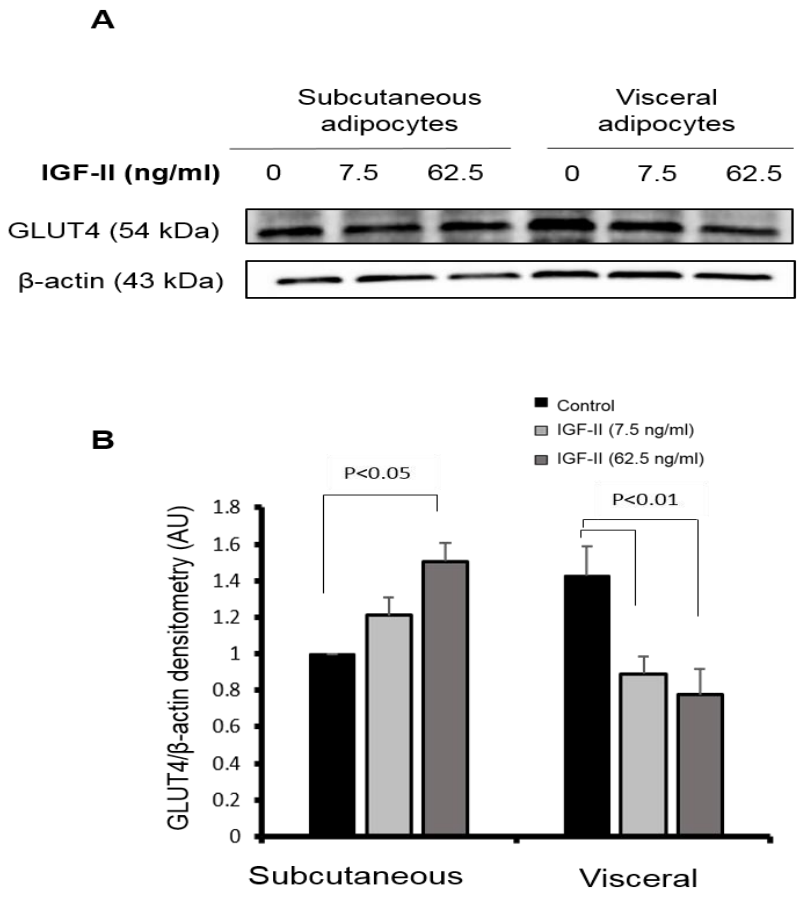


Figure 4-11 Effect of IGF-II on the abundance of the glucose transporter 4 (GLUT4) with differentiation.

(A) Western blotting of paired subcutaneous and visceral preadipocytes differentiated for 14 days with IGF-II (7.5 ng/ml or 62.5 ng/ml) showing GLUT4 protein abundance. (B) Densitometry western blot analysis of (A). β-actin was used to ensure equal loading of samples. Densitometry represents the mean ± SEM of three experimental repeats from three individual biopsies (n=3). Statistical analysis was performed using one-way ANOVA on day 14 of differentiation.

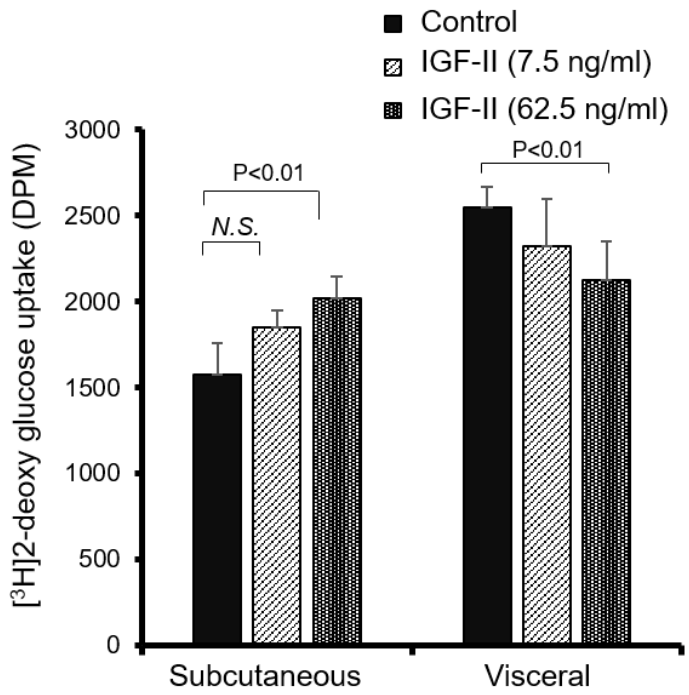


Figure 4-12 Effect of differentiation with IGF-II (7.5 ng/ml, 62.5 ng/ml) on radioactive insulin stimulated glucose uptake.

Subcutaneous and visceral preadipocytes were differentiated in the presence or absence of IGF-II at (7.5 ng/ml) or (62.5 ng/ml), at day 14 of differentiation for 15 minutes. Insulin stimulated H^3 -2deoxy glucose was performed. Data represents the mean ± SEM of three experimental repeats from three individual biopsies (n=3). Statistical analysis was performed using one-way ANOVA on day 14 of differentiation.

4.4.6.3. Effect of IGF-II treatment on fatty acid synthase (FASN) with differentiation in subcutaneous and visceral cultures.

Fatty acid synthase protein abundance was also reduced in visceral adipocytes when differentiated with 7.5 ng/ml IGF-II (0.9vs.1.8; p<0.001) and 62.5 ng/ml IGF-II (0.5 vs. 1.8; p<0.001) and similarly FASN protein abundance was enhanced in subcutaneous adipocytes with both doses of IGF-II; 7.5ng/ml (2.7vs.1.0; P<0.001) and 62.5ng/ml (4.5 vs.1.0; P<0.001) as demonstrated in (Figure 4-13).

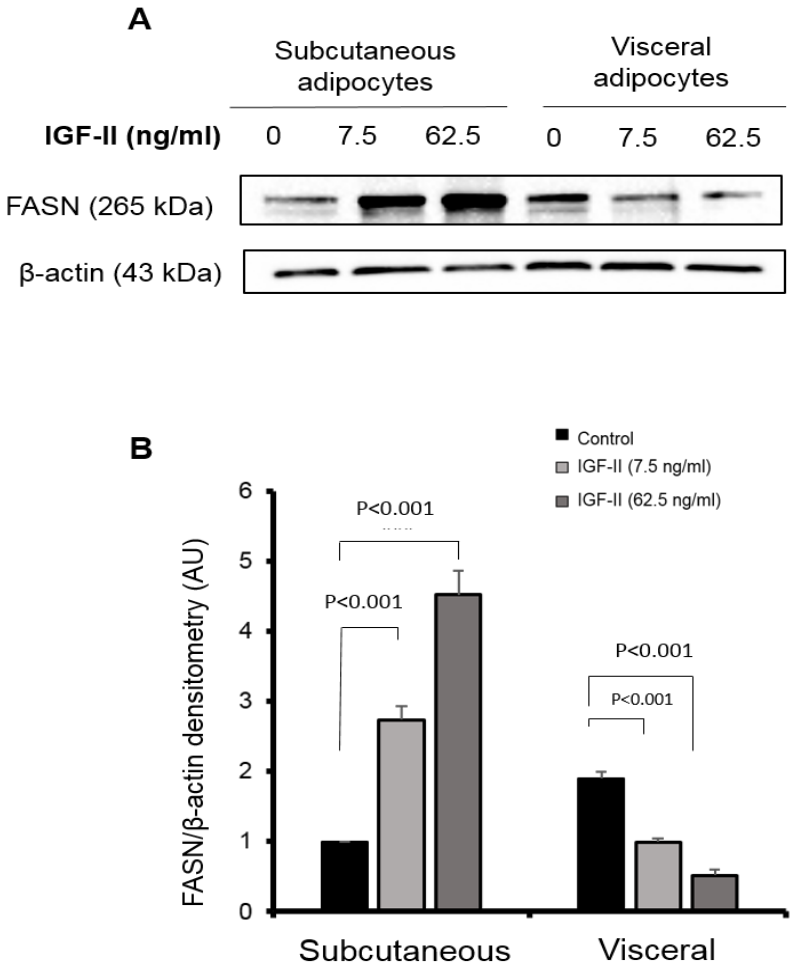


Figure 4-13 Effect of IGF-II on fatty acid synthase (FASN) abundance with differentiation.

Western blotting of paired subcutaneous and visceral preadipocytes differentiated for 14 days with IGF-II (7.5 ng/ml or 62.5 ng/ml) (A) Western blot illustrates FASN protein abundance. (B) Densitometry western blot analysis of (A). β-actin was used as a loading control. Densitometry represents the mean ± SEM of three experimental repeats from three individual biopsies (n=3). Statistical analysis was performed using one-way ANOVA on day 14 of differentiation.

4.4.7. The Effect of acute IGF-II treatment on the expression of insulin receptor and the IGF-IR in subcutaneous and visceral adipocytes.

After investigating IGF-II's role in preadipocyte proliferation and differentiation, we further examined the acute effect of IGF-II on mature differentiated adipocytes to

understand IGF-II actions at the receptor level, these cells being differentiated with normal glucose (5mM/L) differentiation media and collected on day 14. The adipocytes were serum starved for 24 hours followed by 24 hours' treatment with IGF-II (62.5 ng/ml). IGF-II treatment down regulated the mRNA expression of total insulin receptor levels in visceral adipocytes; the expression was normalized to GAPDH reference gene and the relative fold change vs. control was (16.5 vs. 42.07; $p<0.01$). Furthermore, there was also a reduction in total levels of insulin receptor mRNA in subcutaneous adipocytes (15.9 vs.1.0; $P<0.05$) (Figure 4-14).

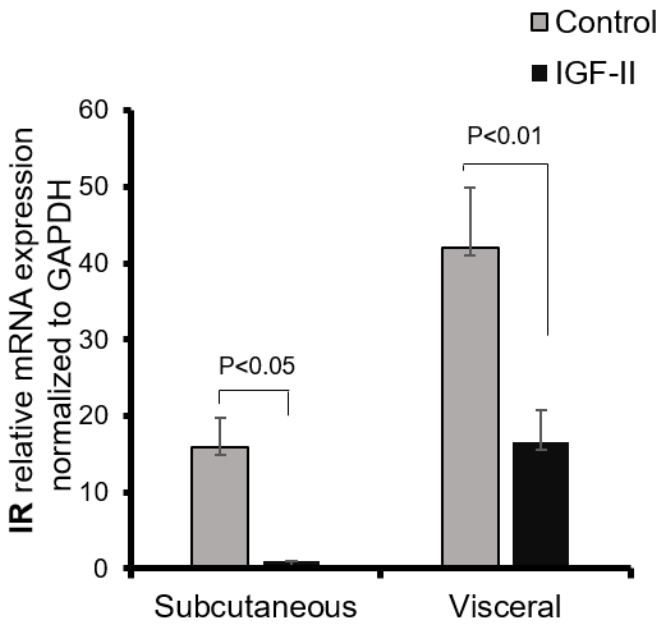


Figure 4-14 :Effect of IGF-II treatment on receptor expression in subcutaneous and visceral differentiated adipocytes.

Relative mRNA expression of the insulin receptor following 24 hours treatment with IGF-II (62.5 ng/ml) was performed using qPCR showing down regulation of the IR in visceral adipocytes. The expression was normalized to the reference gene GAPDH and data are represented as the mean ± SEM of experiments performed in duplicate runs from six individual biopsies (n=6). Statistical analysis was performed using one-way ANOVA.

In terms of the IGF-IR, there were no significant changes in IGF-IR mRNA expression after IGF-II treatment for subcutaneous adipocytes (P=0.098) or visceral adipocytes (p=0.71) (Figure4-15). This was expected as IGF-IR abundance decreased in adipocytes as we showed previously in section (3.4.8).

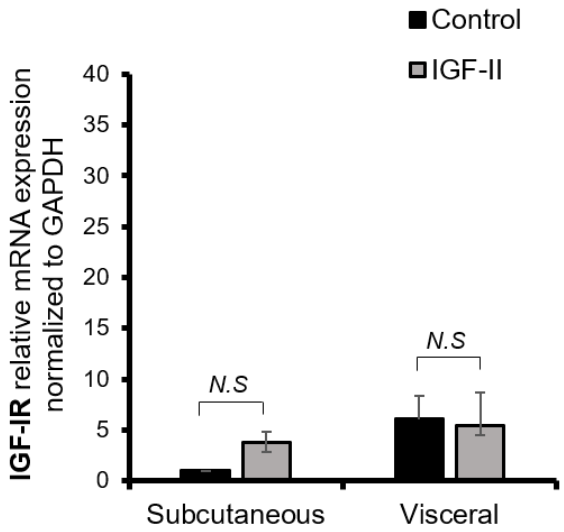


Figure 4-15: Effect of IGF-II treatment on IGFIR receptor expression in differentiated adipocytes.

Relative mRNA expression of the IGF-IR following 24 hours IGF-II (62.5 ng/ml) treatment was performed using qPCR showing no significant changes in receptor expression in subcutaneous and visceral adipocytes. In all experiments, expression was normalized to the reference gene GAPDH and data are represented as the mean ± SEM of experiments performed in duplicate runs from six individual biopsies (n=6). Statistical analysis was performed using one-way ANOVA.

4.4.8. The effect of IGF-II treatment for 24hours on the distribution of insulin receptor isoforms in subcutaneous and visceral adipocytes.

When looking specifically at the insulin receptor isoforms, there was an IGF-II induced down regulation of insulin receptor isoform B in both visceral adipocytes vs. control (5.5 vs. 18.7: P<0.05) (Figure 4-16 B) and subcutaneous adipocytes (2.1vs.14.0: p<00.1) (Figure 4-16 A). However, insulin receptor isoform A was only significantly down regulated in visceral adipocytes (1.4 vs.12.8: P<0.05) consistent with the maintenance of IR-A and maintained sensitivity to IGF-II.

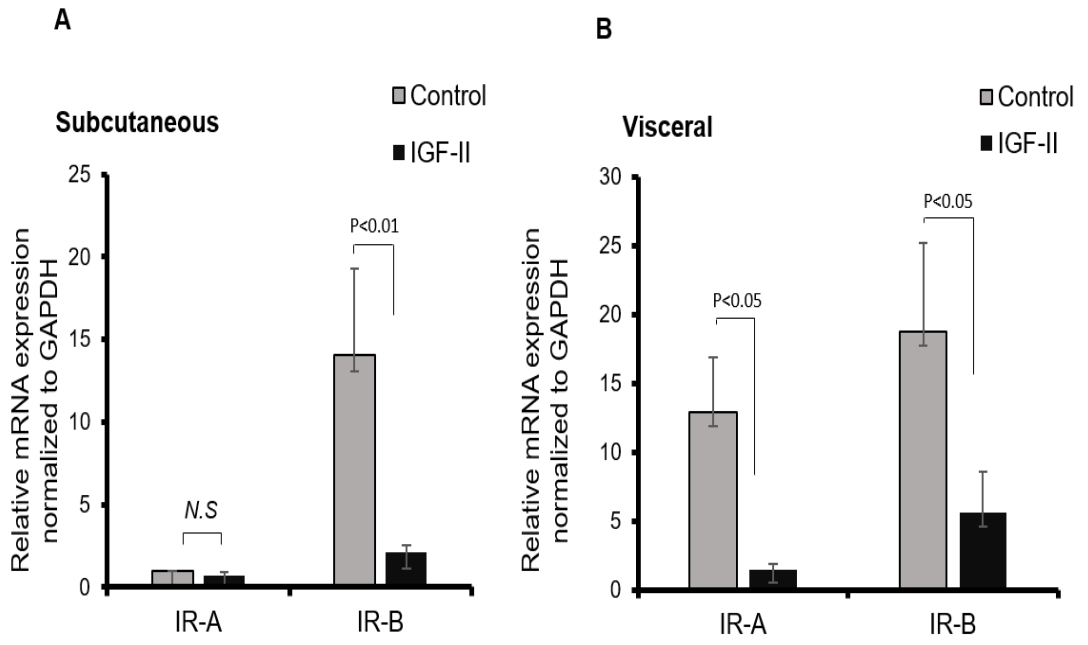


Figure 4-16: Effect of IGF-II treatment on the expression of insulin receptor isoforms in differentiated adipocytes.

Relative mRNA expression following 24 hours treatment with IGF-II (62.5 ng/ml) was performed using qPCR. mRNA expression of the insulin receptor isoforms IR-A and IR-B in (A) subcutaneous and (B) visceral adipocytes showing down regulation of IR-A and IR-B with IGF-II treatment in visceral adipocytes and in IR-B expression in subcutaneous adipocytes. In all experiments, expression was normalized to the reference gene GAPDH and

data are represented as the mean ± SEM of experiments performed in duplicate runs from six individual biopsies (n=6). Statistical analysis was performed using one-way ANOVA.

4.4.9. The effect of 24hours of IGF-II treatment on glucose transporter 4 and glucose uptake in differentiated subcutaneous and visceral adipocytes.

IGF-II treatment markedly down regulated GLUT4 mRNA expression in visceral adipocytes as the fold change in comparison to control was (0.66 vs 8.13; P<0.001). In contrast, IGF-II had a minimal effect on GLUT4 expression in subcutaneous adipocytes (P=0.592) (Figure 4-17).

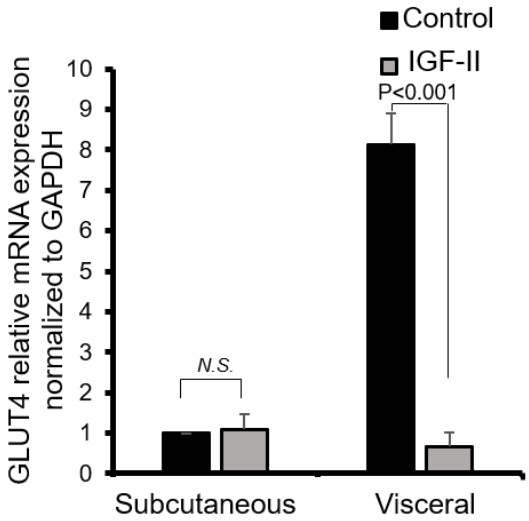


Figure 4-17: Effect of IGF-II treatment on GLUT4 expression in differentiated adipocytes.

Relative mRNA expression of GLUT4 in subcutaneous and visceral adipocytes following 24hours treatment with IGF-II (62.5 ng/ml) measured by qPCR and normalized to GAPDH reference gene. Data are expressed as the mean ± SEM of duplicate runs from three individual biopsies (n=3). Statistical analysis was performed using one-way ANOVA.

To examine if the mRNA expression changes corresponded with protein abundance, we also examined GLUT4 protein abundance for subcutaneous and visceral adipocytes. Visceral GLUT4 protein abundance was also reduced in comparison to control (0.4 vs. 0.8: $P<0.05$) and no significant changes in GLUT4 protein abundance were observed in subcutaneous adipocytes ($P=0.369$) (Figure 4-18)

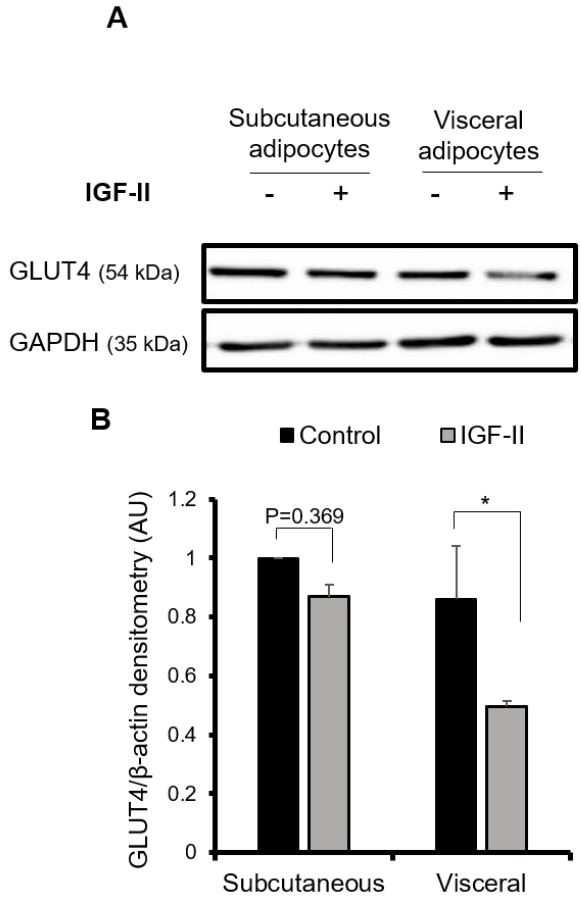


Figure 4-18: Effect of IGF-II on GLUT4 protein abundance in differentiated adipocytes.

(A) Western blot showing GLUT4 abundance; GAPDH was used a loading control. (B) Densitometry analysis of (A) indicating a reduction in GLUT4 protein abundance in visceral adipocytes. Data are expressed as the mean ± SEM and each western blot densitometry is representative of experiments performed in triplicate from three individual biopsies (n=3). (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

The down regulation of GLUT4 in visceral adipocytes was associated with a 33.8 % reduction in 2DG glucose uptake (1580.5 DPM vs 2388.8 DPM; P<0.05). There were no significant changes in glucose uptake with IGF-II treatment in subcutaneous adipocytes (1904.4 DPM vs 1656.3 DPM; P=0.855) (Figure 4-19)

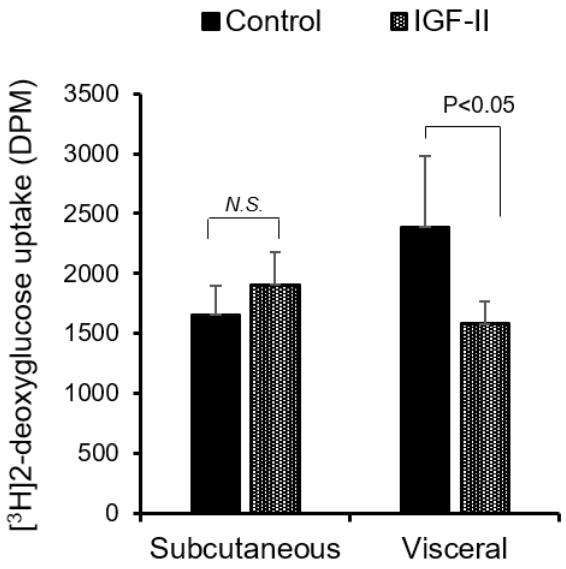


Figure 4-19: Effect of IGF-II treatment on glucose uptake in subcutaneous and visceral differentiated adipocytes.

[^{3}H]2-Deoxy glucose uptake following IGF-II treatment (60 ng/ml) in visceral and subcutaneous adipocytes. Data are expressed as the mean ± SEM of duplicate runs from three individual biopsies (n=3). Statistical analysis was performed using one-way ANOVA.

4.5. Discussion

The post-natal role of IGF-II has been poorly investigated, despite its abundance in the human circulation where it exceeds that of IGF-I and its predominant local production from adipose tissue (Gude, Frystyk et al. 2012). Being a fetal growth promoter, IGF-II *in utero* expression promotes adipogenesis and fat storage during pregnancy (Whitaker and Dietz 1998). Additionally, methylation status of the IGF-II gene at birth has been linked to early childhood weight (Le Stunff, Fallin et al. 2001), and the level of IGF-II in the circulation during childhood has been closely related to fat mass (Ong, Kratzsch et al. 2002). Together these reports indicate a possible regulatory role of IGF-II in adipose tissue.

We investigated IGF-II's actions using paired visceral and subcutaneous fat biopsies and the data suggested that at physiological concentrations, IGF-II has depot-specific actions in terms of promoting preadipocyte growth; IGF-II enhances subcutaneous preadipocyte growth to mature adipocytes and fat deposition but in contrast has a restricting effect on visceral preadipocyte maturation.

IGFs are known mitogenic factors (Jones and Clemmons 1995) , the proliferative effect of IGF-I on preadipocytes is well- established in the 3T3-L1 cell line (Boney, Smith et al. 1998) and in mammalian primary cultures (Wright and Hausman 1995). These studies are consistent with our findings supporting this proliferative effect on 3T3-L1 cell lines. IGF-I also promoted subcutaneous and visceral preadipocyte proliferation with a higher increase in subcutaneous preadipocytes. This might be due to the mitogenic actions of IGF-I reported to be mediated through the IGF-IR (Bäck, Brännmark et al. 2011). Furthermore, a higher level of the IGF-IR was detected in subcutaneous preadipocytes than visceral preadipocytes, section (3.4.8).

IGF-II was also shown to stimulate proliferation in the 3T3-L1 cell line (Siddals, Westwood et al. 2002). However, IGF-II was less effective than IGF-I in promoting 3T3-L1 proliferation. This can be explained since 3T3-L1 preadipocytes almost exclusively express the IGF-IR and IGF-I affinity for IGF-IR is known to be higher than IGF-II and insulin (Varewijck and Janssen 2012). Unlike the 3T3-L1 cell line, studies have indicated that human preadipocytes express both IGF-IR and IR (Bäck, Brännmark et al. 2011). In humans, IGF-II preadipocytes proliferation might also be mediated through the insulin receptor (Morrione, Valentinis et al. 1997), and that IGF-II can stimulates different responses than insulin through the insulin receptor (Morcavallo, Gaspari et al. 2011). Furthermore, insulin receptor isoform expression is different between the two fat depots with the isoform B (IR-B) being predominant in subcutaneous preadipocytes, and the visceral expressing mainly the IR-A form. This might contribute to the proliferative effect of IGF-II as IR-A has a more potent mitogenic effect than IR-B (Belfiore, Malaguarnera et al. 2017). Our findings also indicate and are consistent with other studies that suggest that insulin has less of a mitogenic effect in comparison to IGFs and is more responsible for metabolic action (Siddals, Westwood et al. 2002).

In terms of preadipocyte differentiation , IGF-II stimulated differentiation of adipose tissue isolated from eyelids in humans (Kang, Park et al. 2011) but no human studies focused on the fat-depot specific IGF-II effects. In mammals, similar findings on fat depot differences due to the actions of IGF-II have been reported in fetal baboons, with higher lipid deposition being seen in subcutaneous in comparison to visceral fat (Tchoukalova, Nathanielsz et al. 2009).

The depot-specific pattern of differences in IGF-II has been described more frequently in human genetic studies, with higher methylation of IGF2/H19 imprinting control region *(ICR)* in young adults at the age of 17 being associated with higher IGF-II expression and increases in subcutaneous fat but not increased waist circumference or visceral fat accumulation (Huang, Galati et al. 2012). Furthermore, analysis of the East Hertfordshire cohort study reported that individual variance in the genetic region IGF2-INS-TH is related to body weight. Individuals with an IGF2-INS-TH 5* haplotype that involves the IGF2 Apal A allele together with allele 9 of TH01 and a subset of class I alleles of INS VNTR as a gene cluster, and who are known to have higher IGF-II levels, (O'Dell, Miller et al. 1997) had lower associated waist circumference, hip-to-waist ratio and BMI in comparison to non *5 haplotype individuals (Rodríguez, Gaunt et al. 2004, Rodríguez, Gaunt et al. 2006). Controversially, other studies have suggested that increased IGF-II levels are positively associated with central adiposity (Martin, Holly et al. 2006) and overall weight gain (Roth, Schrager et al. 2002). However, ethnic differences may be important in IGF-II weight-related effects (Fowke, Matthews et al. 2010, Perkins, Murphy et al. 2012). Interestingly, IGF-II regulation of the differentiation markers showed a particular regulation of PPARγ2 , which unlike PPARγ1 is very specific to adipose tissue (Mukherjee, Jow et al. 1997) functioning as an early regulator of the transcription genes needed for adipocytes differentiation (Tontonoz, Hu et al. 1994). This might suggest that IGF-II can regulate preadipocyte growth by influencing the splice variance of PPAR early in the differentiation process. We further investigated how IGF-II treatment affected mRNA expression of the IGF-IR, insulin receptor and its isoforms in adipocytes. Because IGF-IR abundance reduces with differentiation, IGF-II exposure showed a non-significant effect on the IGF-IR in adipocytes. whereas,

IGF-II caused down regulation of total insulin receptor mRNA levels, particularly with respect to IR-A in visceral adipocytes. This was associated with downregulation of GLUT4. IGF-II was reported to activate insulin receptor in adipose cells (Bäck, Brännmark et al. 2011) and with higher affinity to IR-A (Lefebvre, Laville et al. 1998). Furthermore, a supporting report also indicated that IGF-II regulates glucose uptake through the insulin receptor in isolated adipocytes obtained from adult subjects (Sinha, Buchanan et al. 1990) and through IR-A in neonatal hepatocytes (Nevado, Valverde et al. 2006). Because of the higher expression of IR-A in visceral adipocytes in comparison to subcutaneous, this might explain the profound action of IGF-II on visceral in comparison to subcutaneous adipocytes which have less IR-A (Frasca, Pandini et al. 2008). In addition, IGF-II depot-specific actions may be explained by the differences in IGF-II tissue availability. This is regulated by IGF binding proteins (IGFBPs) and the IGF-II receptor (IGF-IIR/M6P) an IGF-II clearance receptor that together regulate IGF-II levels. A difference in IGFBP production between subcutaneous and visceral fat (Gude, Hjortebjerg et al. 2016) has been reported, and a fat-depot difference in IGF-IIR/M6P abundance was also seen in mammals (Tchoukalova, Nathanielsz et al. 2009). Furthermore, fat depot variances in the protease enzymes e.g. the protease pregnancy associated plasma protein-A (PAPP-A) which can regulate IGF binding to the binding proteins (Conover, Harstad et al. 2013) has been indicated to influence fat, as interestingly knocking down PAPP-A in high fat fed mice showed a reduction in visceral fat. Another study indicated that high-fat consumption in PAPP-A knock-down female mice showed an increase in subcutaneous fat and a reduction in retroperitoneal fat (Hill, Arum et al. 2015), however a contrary study also showed no effect (Christians, Bath et al. 2015). This

highlights the importance of investigating IGFs in a fat depot specific manner which can highly affect adipose tissue development.

Overall, our results indicate that IGF-II might have a role as a physiological regulator of preadipocyte growth and metabolism and may play a fat-depot specific role in regulating body fat composition possibly by increasing subcutaneous and reducing visceral fat differentiation.

4.6 Conclusion

Insulin like growth factor -II is an important fetal growth regulator, however little is known about its role after birth. The genetic associations of IGF-II expression and circulating levels with body weight and obesity indicate a regulatory role for IGF-II in adipose tissue. In support of our hypothesis, the results indicate that IGF-II can promote adipose tissue proliferation and differentiation. However, IGF-II acts in a depot-specific manner causing an increase in differentiation of subcutaneous preadipocytes and limiting that in visceral preadipocytes. This was shown by assessment of the differentiation markers PPAR-γ and adiponectin and evaluation of adipocyte maturation indicators (FAS, IR, GLUT4). Furthermore, IGF-II regulated the expression of insulin receptor isoforms in adipocytes and had a more pronounced effect on visceral cells, most likely due to the higher predominance of IR-A in comparison to subcutaneous. In the next chapter, we want to explore if these fat-depot differences were due to changes in IGF-II bio-availability and cellular accessibility.

Chapter 5 : Fat-depot differences in insulin-like growth factor-II (IGF-II) regulation: role of the IGF-II/ mannose-6-phosphate receptor (IGF-IIR/M6P).

5.1 Introduction

The IGF-IIR/M6P receptor is a multifunctional transmembrane glycoprotein that has a unique structure consisting of a large extracellular domain of 2,264 residues and a small intracellular domain consisting of 23 transmembrane residues and a 164-residue cytoplasmic domain (Lemamy, Ndeboko et al. 2016). The binding of IGF-II to the IGF-IIR/M6P receptor does not induce tyrosine kinase activity, but it induces the internalization and lysosomal degradation of the hormone inside the cell (Oka, Rozek et al. 1985). In addition to IGF-II binding, IGF-IIR/M6P has an M6P-bearing site that binds to other ligands including transforming growth factor-β (TGF- β) and plasminogen (Dennis and Rifkin 1991, Leksa, Pfisterer et al. 2012). Human IGF-IIR (IGF-IIR/M6P) has been shown to exhibit bi-allelic expression (Kalscheuer, Mariman et al. 1993, Xu, Goodyer et al. 1993), unlike in rodents where it is only maternally expressed; however, some controversial studies have suggested a similar maternal expression in humans as well (Xu, Goodyer et al. 1993).

Tight maintenance of IGF-II levels is important because dysregulation is not only associated with growth disturbance (Kalscheuer, Mariman et al. 1993) and malignancy (Yamada, De Souza et al. 1997, Oates, Schumaker et al. 1998) but also with metabolic diseases such as diabetes (Jeyaratnaganthan, Højlund et al. 2010). The IGF-IIR/M6P has been shown to act as a growth suppressor and IGF-IIR/M6P gene mutation has been associated with tumour progression, thus the receptor was targeted as a therapeutic approach to treating cancer (Chen, Ge et al. 2002, Hébert 2006, Hu, McCall et al. 2006). However, the roles of both IGF-II and the IGF-IIR/M6P in metabolic regulation are not definitive. Although the IGF-IIR/M6P has a well-

established role as a clearance receptor for IGF-II, it has a less well established role in signalling, but it may facilitate signal transduction in muscle to promote amino acid uptake (Shimizu, Webster et al. 1986) and in pancreatic tissue by affecting insulin exocytosis (Zhang, Tally et al. 1997). However, it still remains to be determined whether IGF-II can induce biological signalling through the IGF-IIR/M6P. IGF-IIR/M6P expression has been identified in adipose tissue in the 3T3-L1 mouse cell line (Shimizu, Torti et al. 1986) and in mammalian tissue (Gardan, Mourot et al. 2008). Nevertheless, limited and conflicting studies have examined IGF-IIR/M6P expression in human adipose tissue (DiGirolamo, Eden et al. 1986, Sinha, Buchanan et al. 1990), mainly focusing on adult adipose tissue with no reports of fat depot-specific comparisons.

Interestingly, circulating IGF-IIR/M6P levels were related to body weight, showing an increase with obesity and a reverse downregulation after weight loss (Oka, Rozek et al. 1985). Furthermore, similar to IGF-II genetic polymorphisms, IGF-IIR/M6P genetic variation has also shown to be related to type 2 diabetes (Chanprasertyothin, Jongjaroenprasert et al. 2015). The association of IGF-IIR/M6P with weight might be due to its role as a regulator of IGF-II, although one study reported no association between variations in IGF-II and IGF-IIR/M6P levels in relation to body weight (Jeyaratnaganthan, Højlund et al. 2010). Furthermore, the IGF-IIR/M6P was shown to be epigenetically regulated as maternal diet altered the expression of the IGF-IIR/M6P in fetal peri-renal adipose tissue, which suggests that the IGF-IIR might have a fat depot-related regulation (Lan, Cretney et al. 2013). This chapter focuses on the regulation of the IGF-IIR/M6P in different fat depots. We hypothesize that IGF-IIR/M6P receptor will show depot-specific differences in abundance and expression

levels, and IGF-IIR/M6P receptor abundance (and subsequent IGF-II secretory levels) will be influenced by insulin treatment.

5.2 Aims and objectives

- Characterise the differences in the expression and abundance of the IGF-IIR/M6P receptor in visceral and subcutaneous preadipocytes and differentiated adipocytes.
- Study the effect of IGF-II treatment on IGF-IIR/M6P abundance in preadipocytes and adipocytes from visceral and subcutaneous fat.
- Study the effect of insulin treatment and different glucose conditions on IGF-IIR/M6P protein abundance in fat depots.
- Examine the impact of altered levels of the IGF-IIR/M6P receptor on secreted levels of IGF-II.
- Study the effect of IGF-II and insulin on IGFBP-3 abundance and secretion from subcutaneous and visceral adipocytes in different glucose conditions.

5.3 Materials and methods

5.3.1 Cell culture

Paired biopsies from subcutaneous and visceral fat were prepared and cultured as described in the methods section (2.2.5). For glucose experiments, preadipocytes and differentiated adipocytes (day 14) were exposed to either high (25 mM/L) or normal glucose (5 mM/L)-containing serum-free media for 24 hours followed by IGF-II (Gropep) or insulin (Novo Nordisk) treatment.

5.3.2. RNA extraction and RNA-cDNA reverse transcription

Total RNA was extracted using Trizol RNA extraction reagent (Invitrogen) as detailed in section (2.6.2): one µg of RNA was reversed transcribed into cDNA using a high capacity RNA-to-cDNA kit (Applied Biosystems) as described in section (2.6.5).

5.3.2 Quantitative PCR (qPCR)

SYBR Green JumpStart Tag ReadyMix (Sigma) was used to perform qPCR using an ABI StepOne Plus Realtime PCR System (Applied Biosystems, 4376600) and following the manufacturer's protocol. Relative mRNA expression was calculated using the $2^{-\Delta Ct}$ method. IGF-IIR/M6P and GAPDH (reference gene) forward and reverse primer sequences are listed in Table (2.6.6).

5.3.3 Western blotting

Western blotting was performed as previously described in section (2.4). Briefly, proteins were run on a 10% SDS-PAGE gel followed by transfer to a nitrocellulose membrane (BioRad) and immunoblotting was performed with the following antibodies: IGF-II receptor was visualised using the IGF-II Receptor/CI-M6PR (D3V8C) from Cell Signaling Technology (1:1000), IGFBP-3 (1:1000, Santa Cruz), GAPDH (1:5000, Millipore) and β-actin (1:10000, Sigma) following the

manufacturer's instructions. The membranes were then incubated with peroxidase-conjugated secondary antibodies (Sigma). Visualization was achieved using SuperSignal West-Dura substrate and Chemidoc XRS + system (BioRad) and quantification using Image J software.

5.3.4. Radioimmunoassay (RIA)

Radioimmunoassays were used to assess IGF-II levels in preadipocytes and adipocytes (at day 14 of differentiation the levels were measured in culture media according to the protocol described in section (2.8)).

5.3.5. Statistical analysis

Data analysis was carried out with the IBM SPSS Statistics 12.0.1 software for Windows, using one-way ANOVA with LSD as a post-hoc test for multiple comparisons (subcutaneous and visceral) and experiments with different glucose conditions. Statistical significance was considered at a p-value of 0.05.

5.4 Results

5.4.1.1 Characterisation of the expression of the IGF-IIR/M6P in subcutaneous and visceral fat depots with differentiation.

First, we investigated IGF-IIR/M6P expression in subcutaneous and visceral preadipocytes and whether receptor expression changed with differentiation. Visceral preadipocytes showed a higher fold change expression of the IGF-IIR/M6P in comparison to subcutaneous preadipocytes (visceral 16.1 vs. 7.7 for subcutaneous; $P < 0.05$). Furthermore, IGF-IIR/M6P mRNA expression decreased with differentiation indicating a higher expression in preadipocytes than adipocytes. These results were observed for both visceral ($P < 0.01$) and subcutaneous ($P < 0.05$) fat depots, as shown in Figure (5-1 A).

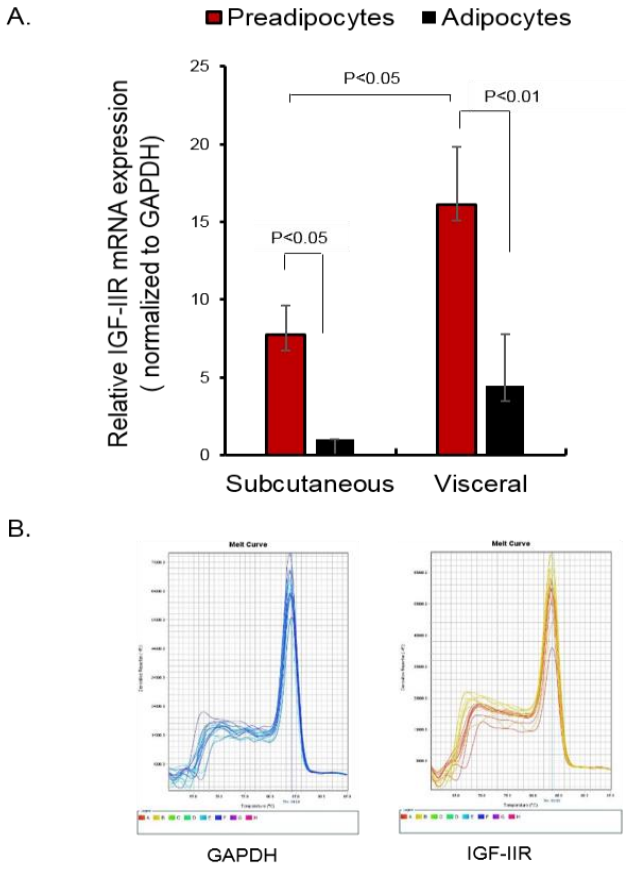


Figure 5-1 Expression of the IGF-IIR/M6P with differentiation in visceral and subcutaneous fat.

A. Relative mRNA expression of the IGF-IIR/M6P in subcutaneous and visceral preadipocytes and differentiated adipocytes. SYBR green-based qPCR was used normalized to the GAPDH reference gene. B. Melting curves of the IGF-IIR/M6P and GAPDH generated during qPCR. Data represent the mean ± SEM of three independent experiments each performed in duplicate. (N = 3)

5.4.1.2 IGF-IIR/M6P protein abundance in subcutaneous and visceral preadipocytes and differentiated adipocytes.

To investigate whether IGF-IIR/M6P protein abundance was comparable to the associated mRNA expression levels, Western blotting was carried out on protein extracts from preadipocytes and differentiated adipocytes (day 14) as indicated in

Figure (5-2 A). IGF-IIR/M6P abundance decreased with differentiation, and the fold change from preadipocytes to adipocytes was 1 vs. 0.44 ($P < 0.05$) in subcutaneous cells and 1.73 vs. 0.48 ($P < 0.001$) in visceral cells. Furthermore, there was a persistent predominance of the IGF-IIR/M6P in visceral preadipocytes in comparison to subcutaneous preadipocytes ($P < 0.05$). There were no fat depot differences in receptor abundance detected in adipocytes.

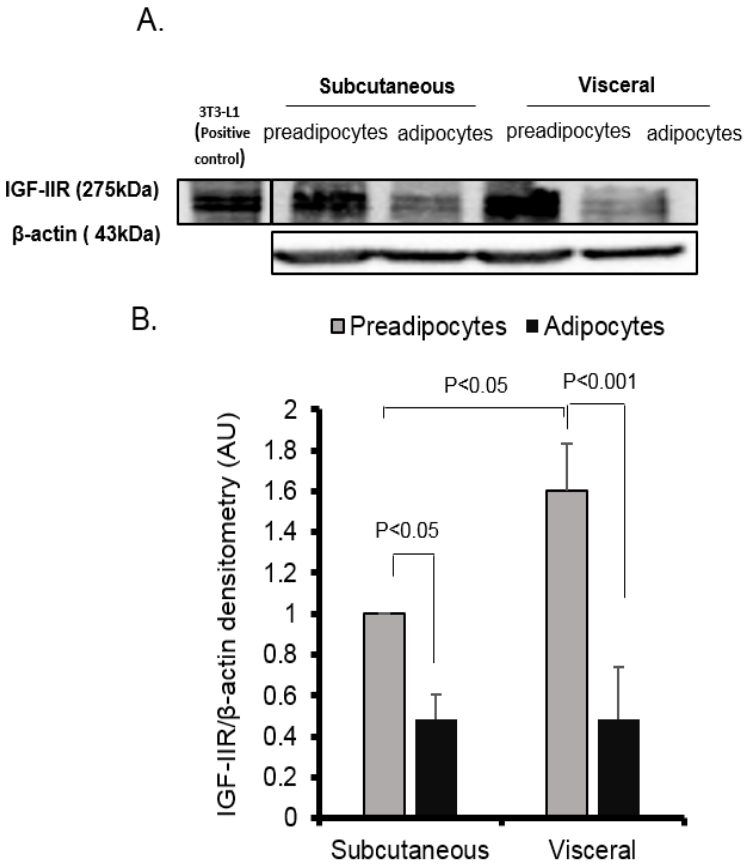


Figure 5-2 Protein abundance of IGF-IIR/M6P in subcutaneous and visceral preadipocytes and adipocytes.

A. Western immunoblot illustrating IGF-IIR/M6P protein abundance. B. Quantification analysis of the Western blot indicating a decrease of IGF-IIR/M6P protein abundance with differentiation in both fat depots. β-actin was used as a reference protein. The 3T3-L1 cell line was used as a positive control. Data are represented as the mean ± SEM. (N=3)

5.4.3 Effect of IGF-II and insulin treatment on the IGF-IIR/M6P in preadipocytes in normal (5 mM/L) and high (25 mM/L) glucose

Preadipocytes were initially used to examine the depot-specific differences in IGF-II on IGF-IIR/M6P levels given the high expression of the latter in these cells. In addition, the effects of insulin on total IGF-IIR/M6P protein abundance was examined in subcutaneous and visceral cells based on previous reports that insulin might influence the distribution of the IGF-IIR/M6P on the cell surface (Sinha, Buchanan et al. 1990) and influence total IGF-IIR/M6P levels assessed by western blotting (Oka, Rozek et al. 1985). Preadipocytes were seeded in normal or high glucose media and serum starved for 24 hrs prior to IGF-II or insulin treatment (60 ng/ml). In normal glucose conditions, IGF-II and insulin had minimal effects on IGF-IIR/M6P abundance in subcutaneous preadipocytes (Figures 5-3 A–B). However, IGF-II treatment in visceral preadipocytes maintained IGF-IIR/M6P abundance whereas insulin showed a downregulation in the IGF-IIR/M6P in comparison (Oka, Rozek et al. 1985). Glucose has been shown previously to regulate the mRNA expression levels of the IGF-IIR/M6P receptor in insulin secreting pancreatic cell lines (Raile, Klammt et al. 2005). Therefore, we examined whether hyperglycaemia alters IGF-II receptor abundance in subcutaneous and visceral cells. Interestingly, high glucose treatment induced an upregulation in IGF-IIR/M6P fold change abundance in subcutaneous preadipocytes in comparison to normal glucose controls (2.6 vs. 1; $P < 0.05$) (Figures 5-3 A–B). IGF-IIR/M6P abundance was persistently high in visceral preadipocytes in both glucose conditions (Figures 5-3 C–D). After IGF-II and insulin treatment, subcutaneous preadipocytes maintained the increased receptor abundance under high glucose conditions in comparison to low glucose. There was also an increase in IGF-IIR/M6P abundance in visceral preadipocytes in high glucose after insulin treatment

($p < 0.05$). These results suggest that high glucose treatment maintains a high IGF-IIR/M6P receptor abundance in both subcutaneous and visceral preadipocytes.

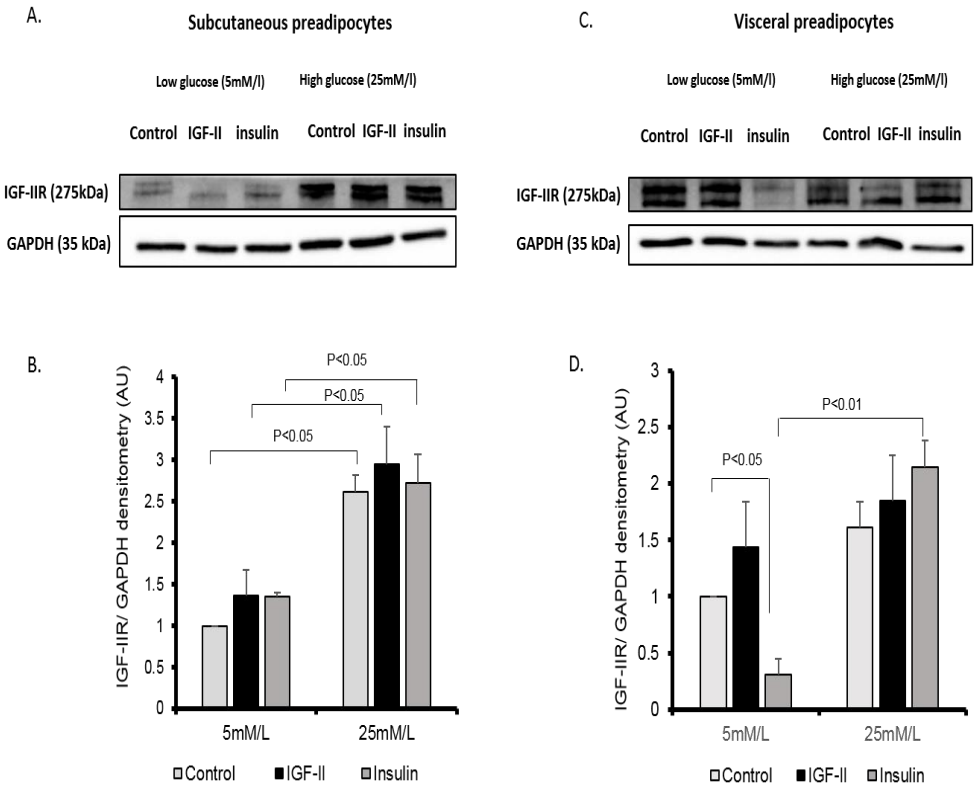


Figure 5-3 Protein abundance of the IGF-IIR/M6P in subcutaneous and visceral preadipocytes in different glucose conditions.

Western blot of A. subcutaneous preadipocytes and C. visceral preadipocytes showing IGF-IIR/M6P abundance. Semi-qualitative densitometry analysis of B. subcutaneous and D. visceral preadipocytes. GAPDH was used as a reference protein. Data are represented as the mean ± SEM, and statistical significance was obtained when $p < 0.05$ ($N = 3$).

5.4.4. Levels of secreted IGF-II from preadipocytes after 24 hours' treatment with insulin in different glucose conditions using radioimmunoassay (RIA)

To determine whether the changes in the levels of the IGF-IIR/M6P receptor will alter IGF-II degradation rates and affect the actual levels of IGF-II in the media, IGF-II

levels were measured using radioimmunoassays in normal glucose (5 mM/L) and high glucose (25 mM/l) conditions. The data indicated no significant difference in IGF-II media levels in either cell type. In subcutaneous preadipocytes, the levels of IGF-II in control vs. after insulin treatment were 5.0 ng/ml vs. 4.1ng/ml, respectively ($p = 0.121$), whereas in visceral preadipocytes secreted IGF-II levels were 5.1ng/ml vs. 4.4 ng/ml, respectively ($p = 0.257$). Figure 5-4 illustrates the changes in secreted IGF-II levels in normal glucose conditions following insulin treatment.

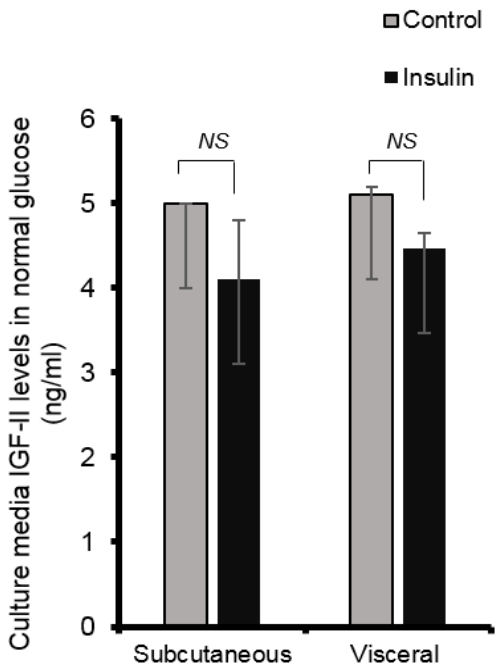


Figure 5-4 Radioimmunoassay measurements of IGF-II levels in subcutaneous and visceral preadipocyte culture media following insulin treatment in normal glucose conditions (5 mM/L).

Conditioned media was obtained from visceral and subcutaneous preadipocytes seeded at $0.2X10^6$ and serum starved for 24 hours followed by insulin treatment. IGF-II levels were measured using radioimmunoassays from three individual experiments performed in duplicate. Data are expressed as the mean ± SEM, and statistical significance was obtained when $p < 0.05$. (N=3)

As IGF-IIR/M6P levels were upregulated in high glucose conditions, we anticipated a corresponding decrease in IGF-II levels due to increased clearance however there were no significant changes in secreted levels of IGF-II levels in subcutaneous preadipocytes (3.4 ng/ml vs. 4.3 ng/ml; P = 0.3) or in visceral preadipocytes (5.03 ng/ml vs. 4.6 ng/ml; P = 0.6) in comparison to controls. These results suggest that changes in IGF-II receptor abundance are not necessarily accompanied by direct changes in IGF-II secreted levels, as illustrated in Figure 5-5.

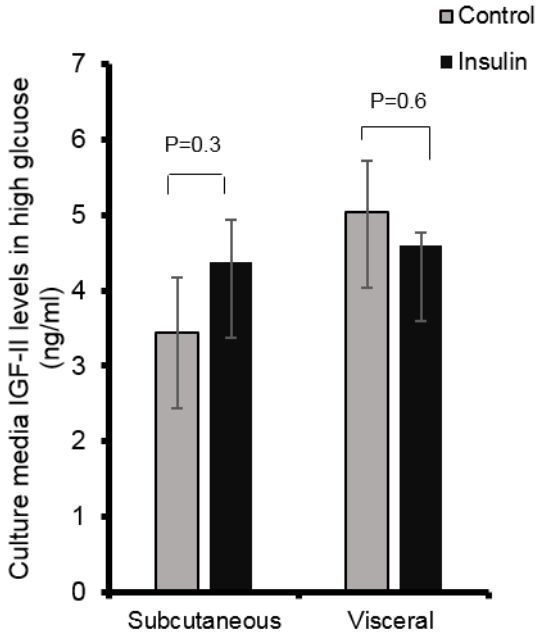


Figure 5-5 Radioimmunoassay measurements of secreted IGF-II in culture media of subcutaneous and visceral preadipocytes in high glucose conditions (25 mM/L).

Conditioned media were obtained from visceral and subcutaneous preadipocytes seeded at $0.2X10^6$ and serum starved for 24 hours followed by insulin treatment in high glucose conditions (25 mM/L). IGF-II levels were measured using radioimmunoassays from three individual biopsies (N=3) run in duplicate. Data are expressed as the mean ± SEM, and statistical significance was obtained when $p < 0.05$.

5.4.5 Effects of IGF-II and insulin treatment on IGF-IIR/M6P levels in adipocytes in different glucose conditions.

The IGF-IIR/M6P receptor was detected at low levels in adipocytes in comparison to preadipocytes under basal stimulation in our model, as indicated in section (5.3.1-2). Given that human studies on isolated adipocytes have shown alterations in IGF-IIR/M6P levels in response to ligand stimulation (Sinha, Buchanan et al. 1990), we further investigated IGF-IIR/M6P levels after IGF-II treatment and whether changes in the physiological environment with high glucose and insulin stimulation will also affect IGF-IIR/M6P levels. As demonstrated in Figure 5-6, insulin was associated with increased IGF-IIR/M6P levels in subcutaneous (2.4 vs. 1.0; $p < 0.01$) and visceral adipocytes (3.3 vs. 1.0; $p < 0.05$) in normal glucose conditions in comparison to controls. Furthermore, IGF-II enhanced IGF-IIR/M6P abundance in visceral adipocytes (3.4 vs. 1.0; $p < 0.05$) with no detectable change in subcutaneous adipocytes. High glucose stimulation was associated with a significant increase in the IGF-IIR/M6P in both fat depots (subcutaneous ($p < 0.01$) and visceral ($p < 0.05$)) in comparison to normal glucose. In subcutaneous adipocytes, hyperglycaemia along with IGF-II treatment (Figures 5-6 A–B) was associated with increased levels of the IGF-IIR/M6P ($P < 0.01$) and insulin treatment maintained this increase. However, IGF-II and insulin treatments had no significant further effect on the increased levels of the IGF-IIR/M6P in hyperglycaemic visceral adipocytes (Figures 5-6 C–D).

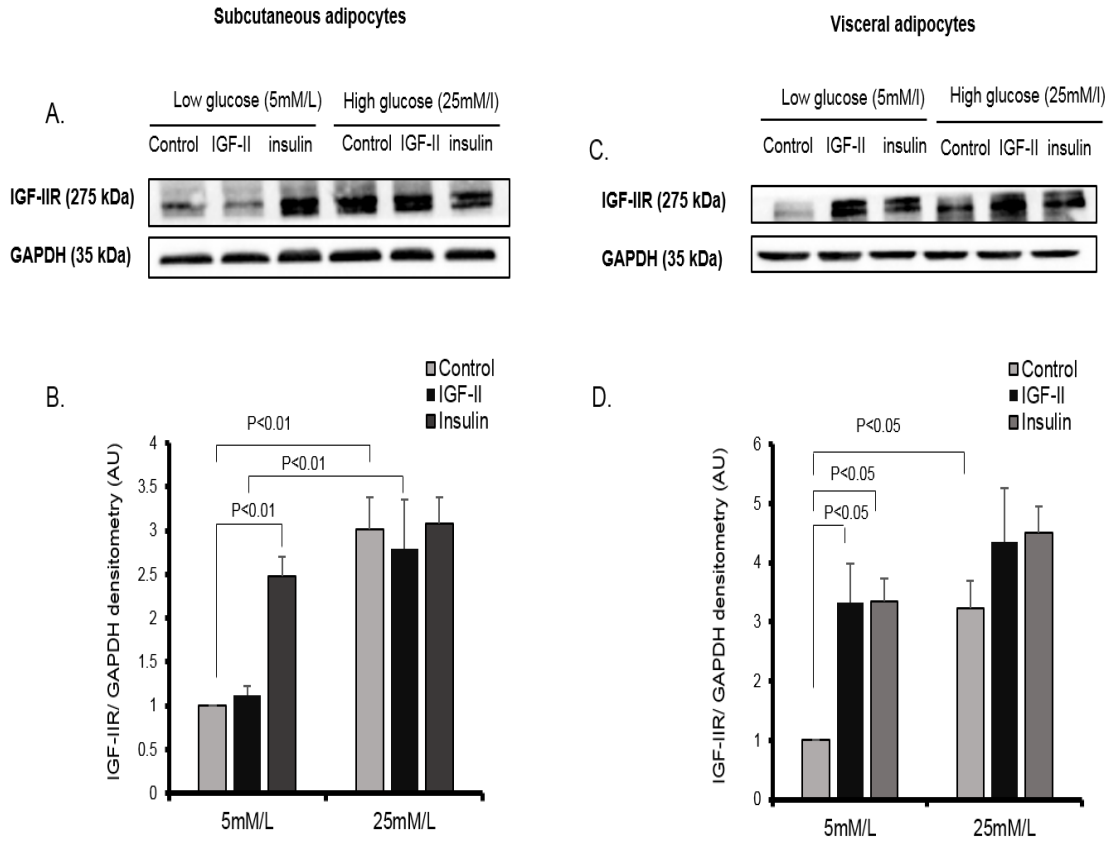


Figure 5-6 Protein abundance of the IGF-IIR/M6P in visceral and subcutaneous adipocytes in different glucose conditions.

Adipocytes at day 14 of differentiation were serum starved for 24 hrs followed by 60 ng/ml of IGF-II or insulin. Western blot of A. subcutaneous adipocytes and C. visceral adipocytes showing IGF-IIR/M6P abundance. Semi-qualitative densitometry analysis of B. subcutaneous and D. visceral adipocyte western blots. GAPDH was used as a reference protein. Data are represented as the mean ± SEM, and statistical significance was obtained when $p < 0.05$ (N = 4).

5.4.6. Levels of secreted IGF-II in adipocytes following insulin treatment in different glucose conditions using radioimmunoassays (RIAs).

We further measured IGF-II secretory levels from subcutaneous and visceral adipocytes following insulin treatment in normal and high glucose conditions. IGF-II levels were not significantly altered after insulin treatment in comparison to controls (2.7ng/ml vs. 3.1ng/ml; $p = 0.90$) in subcutaneous adipocytes and visceral adipocytes

(1.8 ng/ml vs. 2.5 ng/ml; p = 0.22) in normal glucose conditions, as indicated in Figure 5-7. In hyperglycaemic conditions, the levels of IGF-II after insulin treatment also did not show a statistically significant change either in subcutaneous adipocytes (2.8 ng/ml vs. 3.0 ng/ml; p = 0.40) or in visceral adipocytes (4.2 ng/ml vs. 3.9 ng/ml; p = 0.54), as demonstrated in Figure 5-8.

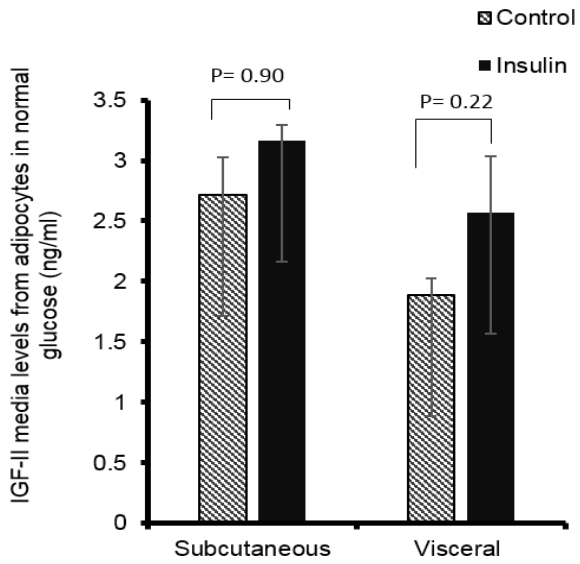


Figure 5-7 IGF-II levels secreted from adipocytes following insulin treatment in normal glucose conditions as determined by RIA.

Subcutaneous and visceral adipocytes at day 14 of differentiation were serum starved for 24 hrs followed by insulin treatment. Conditioned media was subjected to radioimmunoassays in duplicate runs. Result are shown as the mean ± SEM. P-values less than 0.05 were considered statistically significant (N = 4).

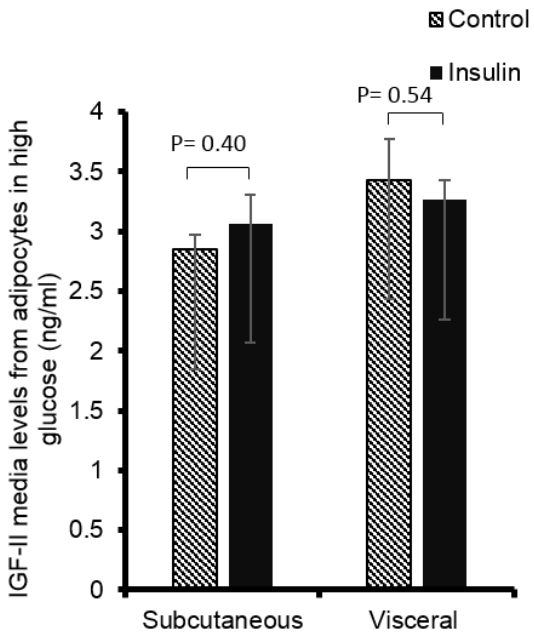


Figure 5-8 IGF-II levels secreted from adipocytes following insulin treatment in high glucose conditions.

Subcutaneous and visceral adipocytes were differentiated in normal glucose for 14 days, cells were serum starved for 24 hrs in high glucose serum-free media followed by insulin treatment. Conditioned media were subjected to radioimmunoassays in duplicate runs. The results are shown as the mean ± SEM. P-values of less than 0.05 were considered statistically significant (N = 4).

5.4.7 Effect of IGF-II and insulin treatment on the level of endogenous IGFBP-3 in visceral and subcutaneous adipocytes.

Since secreted levels of IGF-II were not influenced by altered levels of the IGF-IIR/M6P, we further investigated the effect of insulin and IGF-II treatment on IGFBP-3 abundance in visceral and subcutaneous adipocytes. IGFBP-3 is the most abundant IGF transporter, and it contributes significantly to the regulation of IGFs bioactivities (Baxter 2000). IGFBPs have a higher affinity for IGFs that do the IGF receptors, which may result in the inhibition of IGF receptor activation. Assessment of IGFBP-3 levels

was conducted using western immunoblotting of protein extracts from subcutaneous and visceral adipocytes; intact and fragmented forms of IGFBP-3 were examined in both normal and high glucose conditions. In subcutaneous adipocytes with normal glucose conditions, insulin treatment decreased total endogenous IGFBP-3 levels by reducing intact IGFBP-3 (0.27 vs. 1; $p < 0.01$) and with no change in fragmented IGFBP-3, leading to a decrease in total IGFBP-3 (0.52 vs. 1; $p < 0.01$). The high glucose conditions increased the fragmentation of IGFBP-3 (1.99 vs. 1; $p < 0.05$) and reduced the total IGFBP-3 levels in comparison to high glucose in combination with IGF-II (0.2 vs.1.2; $p < 0.001$) and with insulin treatments (0.1 vs. 1.2; $p < 0.001$), as illustrated in Figure 5-9. The increase in IGFBP-3 fragments in high glucose conditions might lead to a lower affinity of IGFBP-3 for IGF-II and increase IGF-II tissue availability.

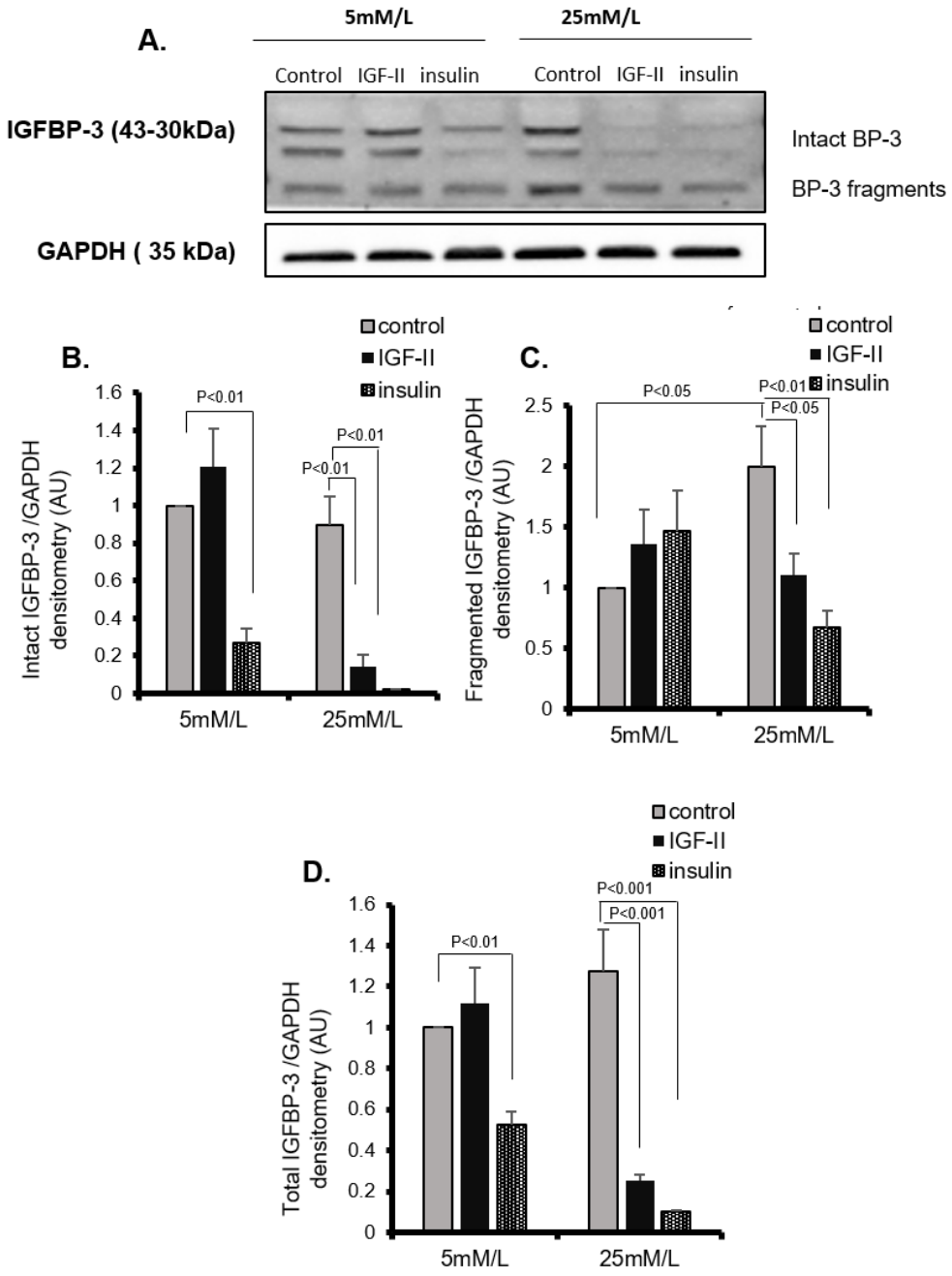


Figure 5-9 Protein abundance of IGFBP-3 in subcutaneous adipocytes in different glucose conditions using Western immunoblotting.

A. Western immunoblot of IGFBP-3 abundance after IGF-II and insulin treatment in normal (5 mM/L) and high glucose (25 mM/L) conditions. Semi-quantitative densitometry analysis of Western blots showing B. intact IGFBP-3 C. fragmented IGFBP-3 and D. total IGFBP-3 protein abundance, normalized to the GAPDH reference protein. The graph represents the mean ± SEM of three independent repeats (N = 3). Statistical analysis was considered significant at $P < 0.05$.

In visceral adipocytes, there was no change in intact IGFBP-3 after IGF-II (1.5 vs. 1; $p = 0.10$) and insulin (1.4 vs. 1; $p = 0.23$) treatments in normal glucose conditions. However, there was a significant increase in fragmented IGFBP-3 following treatment with IGF-II (1.3 vs. 0.4; $P < 0.01$) and insulin (0.84 vs. 0.4; $p < 0.05$), and a small increase in total IGFBP-3, as indicated in Figures (5-10 A–D). In high glucose conditions, intact IGFBP-3 was reduced in comparison to that in normal glucose conditions (0.04 vs. 1, respectively; $p < 0.01$) and following IGF-II (0.3 vs. 1.5, respectively; $P < 0.01$) and insulin (0.2 vs. 1.4, respectively; $P < 0.01$) treatments. IGFBP-3 proteolysis increased after IGF-II (0.9 vs. 0.3; $p < 0.05$) and insulin (1.0 vs. 0.3; $p < 0.01$) treatments in comparison to controls in high glucose, which might cause an increase in local IGF tissue availability. Total IGFBP-3 was also increased in high glucose after IGF-II and insulin treatments ($p < 0.05$), and interestingly total IGFBP-3 was reduced in comparison to that in normal glucose conditions (0.3 vs.1.3; $p < 0.05$) as shown in Figure (5-10 D).

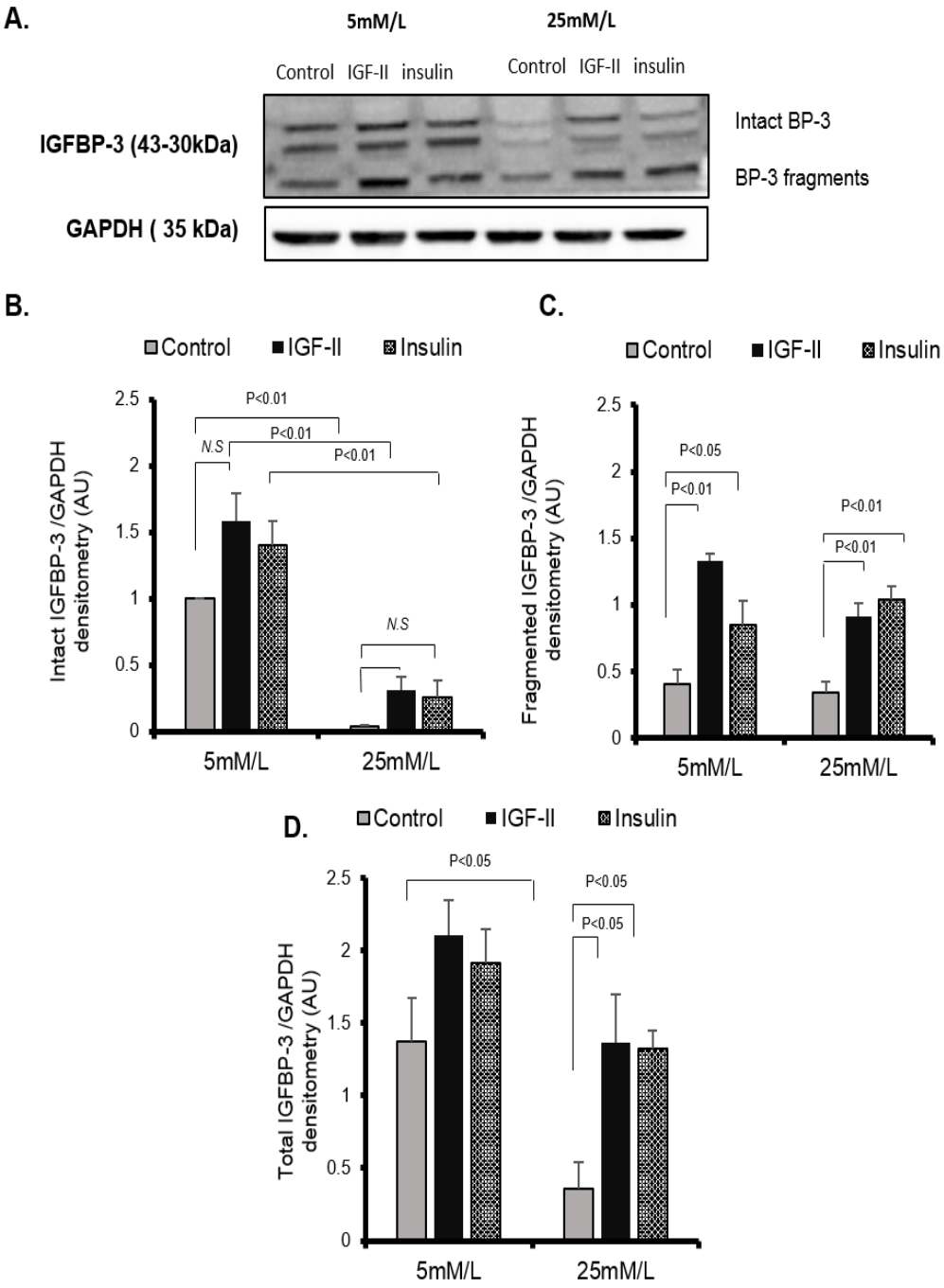


Figure 5-10 Protein abundance of IGFBP-3 in visceral adipocytes following IGF-II, insulin and high glucose treatments.

A. Immunoblot of IGFBP-3 showing intact and fragmented BP-3. Semi-quantitative densitometry analysis in normal (5 mM/L) and high (25 mM/L) glucose conditions after 24 hrs IGF-II and insulin treatment of B. intact IGFBP-3 C. fragmented IGFBP-3 and C. total IGFBP-3. Normalized to reference protein GAPDH. The graph represents the mean ± SEM from three individual biopsies (N=3). A P-value of less than 0.05 was considered significant.

IGFBP-3 levels were also measured using radioimmunoassay in subcutaneous and visceral adipocytes in conditioned media to confirm IGFBP-3 production levels. It is important to note that the IGFBP-3 radioimmunoassay detects both intact and fragmented IGFBP-3.

In subcutaneous adipocytes, IGF-II had a minimal effect on IGFBP-3 levels in normal glucose conditions. There were no changes in the pattern of IGFBP-3 secretion in normal and high glucose conditions after insulin treatment, as shown in Figure (5-11 A). In visceral adipocytes, there were no significant changes in IGFBP-3 secretion in normal glucose conditions after IGF-II ($p = 0.9$) and insulin ($p = 0.5$) treatments in comparison to controls. There was a 1.5-fold decrease in IGFBP-3 secretion after exposure to high glucose ($p < 0.05$). Furthermore, IGF-II treatment significantly increased IGFBP-3 secretion in high glucose ($p < 0.05$) in comparison to high glucose controls, as demonstrated in Figure (5-11 B). In high glucose, this reduction was mainly due to intact IGFBP-3 rather than fragmented IGFBP-3 which might increase the availability of IGF-II.

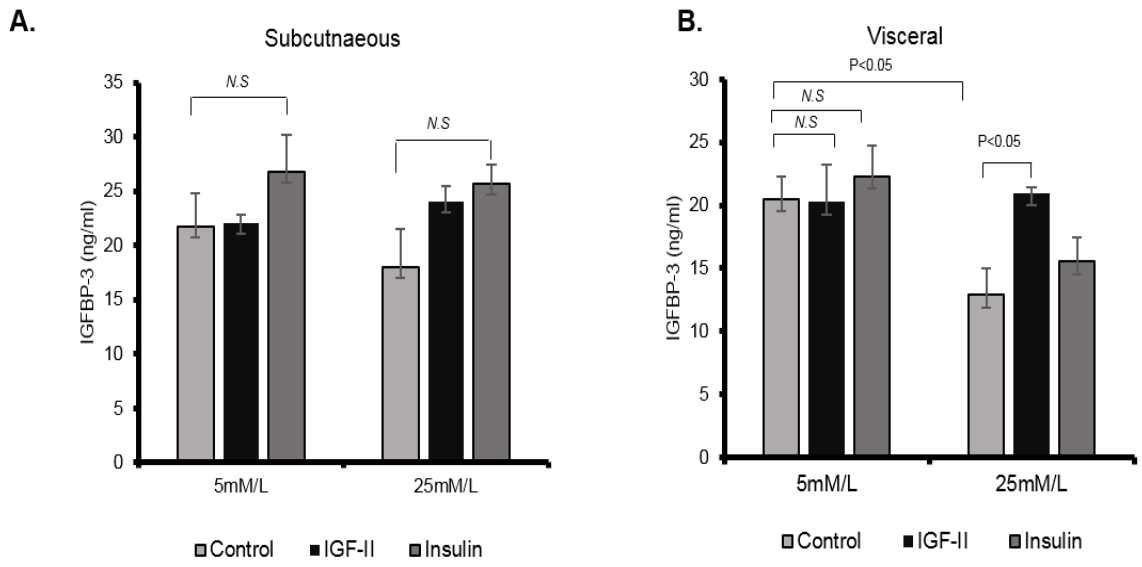


Figure 5-11 Secreted levels of IGFBP- from subcutaneous and visceral adipocytes measured by radioimmunoassay (RIA).

Supernatants obtained from subcutaneous and visceral adipocytes were subjected to radioimmunoassays from three individual biopsies performed in duplicate. Data are expressed as the mean ± SEM. Results from statistical analysis were considered significant at $P < 0.05$. (N =3)

5.5 Discussion

This chapter focused on the regulation of IGF-IIR/M6P in adipose tissue, particularly investigating any fat depot differences in IGF-IIR/M6P levels that act as an important regulator of IGF-II availability and function in multiple tissues (Hari, Pierce et al. 1987, Han, Luan et al. 2011). Given the limited literature investigating IGF-IIR/M6P levels in adipose tissue, our data indicate that the IGF-IIR/M6P was detected in preadipocytes and decreased in abundance during differentiation. This expression pattern was consistent with that described in previous studies using 3T3-L1 cell lines (Shimizu, Torti et al. 1986) and from mammalian studies examining the subcutaneous fat obtained from porcine samples (Gardan, Mourot et al. 2008). In humans however, IGF-IIR/M6P expression and protein abundance were reported in cultured fibroblast cells (Ryu, Hwang et al. 2007) but studies were more controversial in detecting the IGF-IIR/M6P in mature adipocytes. The IGF-IIR/M6P was found in isolated subcutaneous adipocytes obtained from adult individuals (Shimizu, Torti et al. 1986) however others failed to detect the IGF-IIR/M6P in adipocytes from murine cells (Ryu, Hwang et al. 2007) and adult human fat cells (Gardan, Mourot et al. 2008). Our results indicated that IGF-IIR/M6P expression and abundance was minimally detected in adipocytes and was less than that in preadipocytes.

Further comparisons of IGF-IIR/M6P fat-depot levels showed a predominance of the IGF-IIR/M6P in visceral preadipocytes in comparison to subcutaneous as indicated by mRNA expression and protein abundance. This pattern was not maintained in adipocytes, mostly due to the lower levels of the IGF-IIR/M6P with differentiation.

As far as we know, there are no studies comparing the levels of the IGF-IIR/M6P levels between human fat-depots. However, higher levels of the IGF-IIR/M6P were identified in visceral than in subcutaneous fat depots of fetal baboon adipose tissue (Tchoukalova, Nathanielsz et al. 2009). The higher IGF-IIR/M6P levels in preadipocytes would suggest a role in regulating preadipocyte differentiation; however, IGF-IIR/M6P knockdown in preadipocytes did not affect the differentiation process (Kang, Park et al. 2011).

Following IGF-IIR/M6P characterization, we investigated whether IGF-IIR/M6P levels were affected by insulin stimulation. Our results indicate that insulin induced an increase in IGF-IIR/M6P abundance and this is consistent with previous studies in murine adipocytes (Appell, Simpson et al. 1988) and in humans tissue using subcutaneous isolated adipocytes (Sinha, Buchanan et al. 1990). The mechanism by which insulin affects the redistribution of the IGF-IIR/M6P is still not clear, and the involvement of multiple kinases has been suggested (Kiess, Yang et al. 1994). Our results showed a more predominant change in receptor distribution due to insulin treatment in adipocytes with a minimal effect on preadipocytes. This may synchronize with the suggestion that IGF-IIR/M6P is recycled with GLUT4 'which is found in higher abundance in mature cells' and they traffic together in sequestered vesicles that fuse to the plasma membrane after insulin stimulation (Wabitsch, Heinze et al. 2000, Slomiany and Rosenzweig 2004). In contrast, this study suggested that insulin administration did not affect IGF-IIR/M6P levels however, methodological differences may have accounted for this discrepancy as the soluble IGF-IIR/M6P was measured in the circulation and not in an *in vitro* setting (Jeyaratnaganthan, Højlund et al. 2010).

Our data showed that hyperglycaemia also caused an increase in IGF-IIR/M6P abundance basally and with ligand stimulation. This is consistent with other studies indicating that glucose can stimulate phosphorylation, which enhances IGF-IIR/M6P receptor levels (Scharf, Schmidt-Sandte et al. 1995). Effects of glucose on the IGF-IIR/M6P were also previously reported using RINm5F and HIT insulin-secreting pancreatic and leukaemia cell lines (Yandell, Dunbar et al. 1999).

As a lysosomal clearance receptor, the IGF-IIR/M6P regulates circulating IGF-II levels. In genetic modulation studies, mice lacking the IGF-IIR/M6P had high circulating levels of IGF-II due to the reduction in the clearance receptor, and this was associated with fetal over-growth (Lau, Stewart et al. 1994). Therefore, we further evaluated secreted levels of IGF-II and explored whether alterations in IGF-IIR/M6P abundance would subsequently affect IGF-II levels. Our data indicated that insulin-induced changes in IGF-IIR/M6P levels were not directly associated with changes in IGF-II media levels over the timeframe of our study. It is possible that although insulin induced the translocation of IGF-IIR/M6P to the cell surface, this might not be associated with an increase in IGF-II clearance via its own receptor (Oka, Mottola et al. 1984, Wardzala, Simpson et al. 1984). However, insulin treatment has been reported to cause an increase in the binding of IGF-II to its conjugate receptor in adipocytes (King, Rechler et al. 1982).

To further investigate IGF-II tissue availability, we examined IGFBP-3 as a major regulator of IGF-II activity. In addition, to being the most abundant circulating binding protein with effects on mediating IGF access to the targeted tissue, IGFBP-3 is also secreted locally by many cells (Scharf, Schmidt-Sandte et al. 1995, Wabitsch, Heinze et al. 2000, Slomiany and Rosenzweig 2004). IGFBP-3 has been strongly associated with adipogenesis (Chan, Schedlich et al. 2009, Baxter 2013). IGFBP-3 is minimally

secreted by preadipocytes (Grohmann, Sabin et al. 2005); however, its levels increase in human adipocytes as the cells differentiate (Wabitsch, Heinze et al. 2000). Therefore, we further investigated the role of IGFBP-3 in adipocyte regulation in a depot-specific manner and whether exogenous treatment of IGF-II and insulin or a change in glucose conditions may alter IGFBP-3 production or fragmentation.

In normal glucose, our results showed a decrease in intact IGFBP-3 in subcutaneous adipocytes with insulin treatment, and this was associated with a subsequent decrease in total IGFBP-3. In high glucose, there was an associated increase in fragments of IGFBP-3 with no change in intact IGFBP-3, suggesting that high glucose by itself seems to increase the fragmentation of IGFBP-3. Insulin treatment still reduced total levels of IGFBP-3 in high glucose.

In visceral adipocytes, normal glucose was associated with an increase in fragmentation of IGFBP-3 with no significant change in intact IGFBP-3. In high glucose, intact IGFBP-3 levels were reduced compared with normal glucose with only an increase in fragmentation leading to a reduction in total IGFBP-3 in comparison to normal glucose. Increased fragmentation of IGFBP-3 has been associated with an exposed NH_2 terminal in IGFBP-3, which leads to a reduced affinity for IGF-II. Such fragmentation will affect IGF-II availability and could cause a dysregulation in insulin receptor signalling making it more predisposed to metabolic disturbances associated with obesity and diabetes (Kim 2013). Intact IGFBP-3 has an unexposed NH_2 terminal with an increased affinity for IGFs, and interestingly, intact IGFBP-3 has been associated with an IGF-independent anti-inflammatory action (Mohanraj, Kim et al. 2013).

As our data indicate that fragmented IGFBP-3, which has less affinity for IGF binding, was higher in visceral than in subcutaneous adipocytes with IGF-II treatment.

The increase in cleavage might be associated with increased bioavailability of IGF-II due to less binding to IGFBP-3 (Baxter 2000). Furthermore, hyperglycaemia seems to increase the protease activity acting on IGFBP-3 leading to altered availability of IGF-II. (Conover 1991). Interestingly, an increase in IGFBP-3 protease activity and a corresponding reduction in intact IGFBP-3 was correlated with obesity and waist circumference, as observed in our study after culturing cells in high glucose conditions in visceral cells (Mohanraj, Kim et al. 2013). The reduction in IGFBP-3 secretory levels was observed in visceral adipocytes after high glucose treatment with no evident changes in subcutaneous adipocytes. Only subcutaneous IGFBP-3 production was measured in other studies: they showed an increase in IGFBP-3 with insulin treatment, however in contrast to our study this was performed using adult samples (Wabitsch, Heinze et al. 2000) . Measuring intact and fragmented IGFBP-3 separately may be interesting and could provide additional understanding to the role of IGFBP-3 in the bioavailability of IGF-II.

In summary fat depot differences in IGFBP-3 were observed that may influence the bioavailability of IGF-II. This provides evidence suggesting other members of the IGFBP family and secreted proteases may play a role in a fat-depot specific manner. In fact, recent reports have shown that IGFBP-4 and its specific protease, pregnancy associated plasma protein–A (PAPP-A), were differentially secreted between subcutaneous and visceral fat that may influence the tissues' response to the growth factors (Gude, Hjortebjerg et al. 2016, Hjortebjerg, Berryman et al. 2018).

5.6 Conclusion

IGF-II bio-availability is precisely regulated and an important IGF-II regulator is the IGF-IIR/M6P clearance receptor. In this chapter we investigated IGF-IIR/M6P receptor abundance in subcutaneous and visceral preadipocytes and adipocytes and examined factors that might alter IGF-IIR/M6P levels in a fat-depot specific manner. Our data indicate that IGF-IIR/M6P abundance decreases with differentiation and also differs in abundance between subcutaneous and visceral preadipocytes. Although insulin treatment affected the abundance of the IGF-IIR/M6P, this did not alter the clearance of secreted IGF-II. Hyperglycaemia increased IGF-IIR/M6P receptor abundance in subcutaneous and visceral cells, and increased IGFBP-3 fragmentation causing a potential increase in IGF-II tissue availability. Future research is required to further understand fat-depot specific differences in IGF tissue bioavailability and subsequent function.

Chapter 6: Correlation between prepubertal IGF-II and post-pubertal fat distribution: findings from the Avon Longitudinal Study of Parents and Children (ALSPAC)

6.1 Introduction

Circulating levels of IGFs are related to many physiological and pathological conditions in relation to tissue growth (Johansson, Baglietto et al. 2004, Clemmons 2012). The biological effects of IGFs are tightly regulated in the circulation and, in the case of IGF-I is hormonally regulated by growth hormone (GH). The GH–IGF-I axis has an established role in the development of specific tissues and body composition; for example, reduced GH levels are associated with increased body fat and reduced lean mass (Veldhuis, Liem et al. 1995). GH indirectly acts via IGF-I to promote preadipocyte proliferation and differentiation (Smith, Wise et al. 1988). and circulating IGF-I levels are associated with body weight (Merimee, Zapf et al. 1982, Yamamoto and Kato 1993), and dysregulation of GH–IGF-I levels are linked with obesity (Maccario, Ramunni et al. 1999). IGF-I levels were also shown to be related to the arrangement of body fat deposition in obese individuals (Rasmussen, Frystyk et al. 1994).

The relationship between postnatal circulating IGF-II levels and body composition has not been extensively described in the literature, presumably because IGF-II is considered to be a prenatal growth factor with sustained, high levels throughout pregnancy (Constância, Hemberger et al. 2002). However, IGF-II is also maintained postnatally and remains high throughout adult life, while IGF-I peaks in puberty and reduces with ageing (Zapf and Froesch 1986). IGF-II is not regulated by GH; however, it has a restricted genetic expression which regulates the level of IGF-II in the circulation (Giannoukakis, Deal et al. 1993). Polymorphisms in *IGF-II* are associated with differences in circulating IGF-II levels; for example, individuals with the IGF-II Apal AA genotype have higher circulating IGF-II levels than those with the IGF-II

Apal GG genotype. Intriguingly, Apal AA individuals have a lower weight than those with the Apal GG genotype (O'Dell, Miller et al. 1997). Baseline circulating levels of IGF-II in the blood predict, future weight gain (Sandhu, Gibson et al. 2003), and IGF-II levels are reversible following weight loss. The role of IGF-II in regulating body weight is not clear; however, these findings indicate that circulating IGF-II levels may be a prognostic biomarker for obesity.

The metabolic risk of obesity is not necessarily related to overall body weight, as this measurement encompasses lean mass and subcutaneous fat that may be protective against metabolic disease (Münzer, Harman et al. 2001). Most IGF-II studies so far have focused on correlations with BMI or body weight, however a more recent report suggested more fat-depot specific associations with IGF-II in adults (Song, Ernst et al. 2018). Dual-energy X-ray absorptiometry (DXA) scans measure body fat compartments in a cost-effective and non-invasive manner, thus making this approach suitable for large population studies (Lee and Gallagher 2008, Shepherd, Ng et al. 2017). The Avon Longitudinal Study of Parents and Children (ALSPAC) is a large longitudinal study that performed DXA scans and measured IGF-II plasma levels at several time points, thus making it a valuable source in which to investigate the correlation between IGF-II plasma levels in early life and the distribution of body fat at puberty (Fraser, Macdonald-Wallis et al. 2012).

6.2 Aim

Using the ALSPAC cohort, this study examined the association between prepubertal serum IGF-II levels and pubertal fat distribution using DXA scan measurements, particularly the levels of trunk and peripheral fat mass at age 15 and 17. We hypothesised, based on the laboratory work in this thesis, that prepubertal serum IGF-II levels would associate with body fat distribution, negatively correlating with pubertal trunk fat mass.

6.3 Study methods

6.3.1 Study subjects

ALSPAC is a prospective observational study conducted in Bristol, United Kingdom. The main aim of this trans-generational study is to explore how the environment and genetics impact on health and growth of individuals (Fraser, Macdonald-Wallis et al. 2012). The ALSPAC study recruited 14,541 pregnant women who were expected to give birth between April 1991 and December 1992. The number of children born was 14,062, out of which 13,988 were alive at 1 year of age. A group of children who were born in the last 6 months of the study (approximately 10% of the cohort), were invited to participate in an ALSPAC sub-study called 'Children in Focus'. This sub-cohort comprised 1,432 children who were brought to the research clinics by their parents and were evaluated from between 4 months and 5 years old. By the age of 7, all children from the cohort had been invited to the clinics. An additional invitation was sent out to all eligible participants who failed to join the initial study, which added an extra 713 children to the study and enhanced the total number of children who were alive at age 1 to 14,701. Further details of the sample size are provided on the study website (www.bristol.ac.uk/alspac) (Golding, Pembrey et al. 2001, Boyd, Golding et al. 2013).

Extensive follow-up was performed at birth, yearly from age 7–13 and then at age 15 and 17. Data were collected in the form of questionnaires, clinical assessments, biological samples and DNA.

6.3.2 Ethics

Ethical approval was gained from the ALSPAC Ethics and Law Committee and the local research ethics committees. This study proposal approval number was B2840.

6.3.3 Exposures

IGF-II measured in the cord blood and at 61 months of age (~5 years) was used for analysis. Cord blood was collected by an assigned midwife and a non-fasting venous blood sample was obtained by a research nurse at the 61 month's clinic. The blood samples were centrifuged and stored at −70ºC. IGF-II levels were determined using the direct enzyme-linked immunosorbent assay (Diagnostic Systems Laboratories, Inc., Sinsheim, Germany) technique, following the separation of IGF-II from its binding complexes. The lower limit of detection of the assay was 40 ng/ml, the inter- and intra-assay coefficients of variation were <10% and the average detection was 300–1200 ng/ml.

6.3.4 Outcomes

Post-pubertal body fat distribution data at age 15 were obtained from teen focus 3 (TF3) research clinic participants who attended between October 2006 and November 2008. Data at age 17 were obtained from teen focus 4 (F17) research clinic participants attending between December 2008 and June 2011. Fat mass was measured using DXA scan data. DXA scan measurements (Lunar Prodigy DXA scanner; GE Medical Systems, Madison, WI, USA) in TF3 (mean age = 15.5 years) and F17 (mean age = 17.8 years) participants were performed during research clinic visits. DXA uses two X-ray beams of different energies that pass through tissue and are attenuated to different degrees according to the nature of the tissue (Rothney, Brychta et al. 2009). It provides detailed measures of total body fat, trunk body fat, peripheral body fat (arms, legs, android and gynoid) and lean mass. Figure 6-1 shows a flowchart of the participants from the ALSPAC study involved in the analysis.

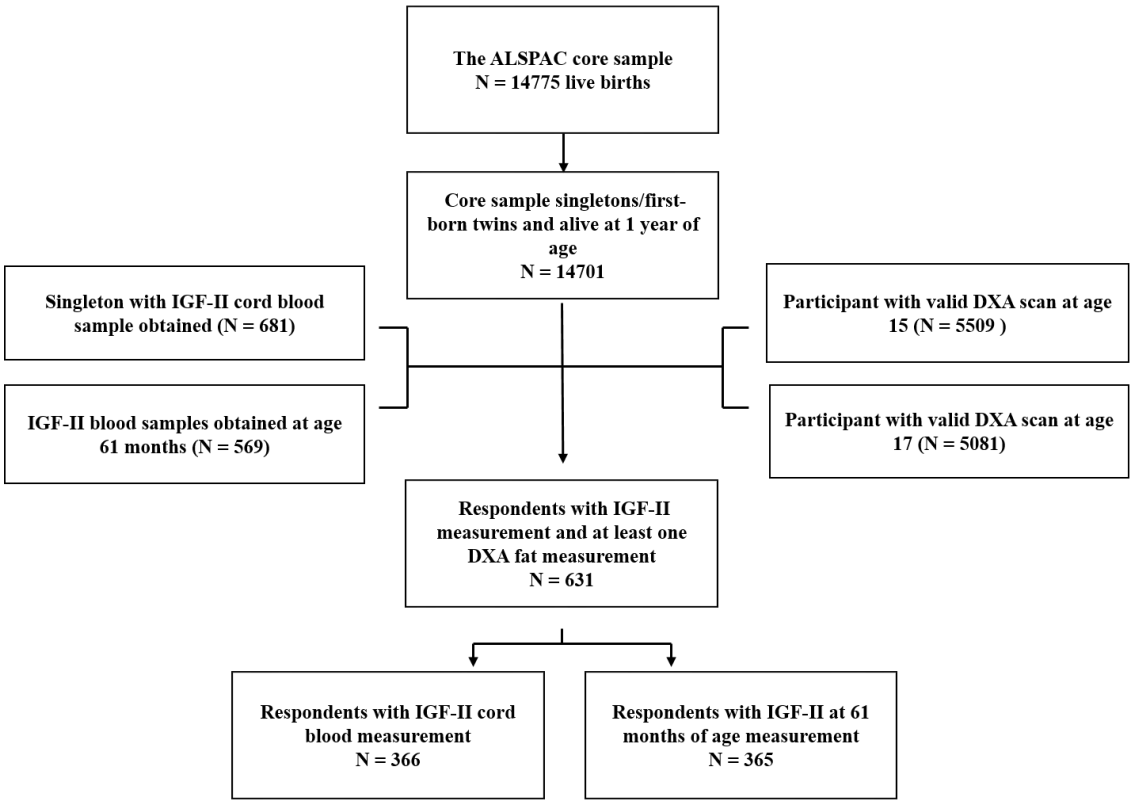


Figure 6-1: Illustration of ALSPAC study numbers included in the final analyses.

Flowchart showing the core sample, singletons or first-born twins, respondents with cord blood samples, respondents with blood samples obtained at 61 months, respondents with DXA scan measurements at 15 and 17 years and respondents with IGF-II and DXA scan measurements.

6.3.5 Confounders

- **Anthropometric measurements:** infant weight was measured immediately after birth by the assigned midwife and recorded in the hospital notes. Height and weight were measured when the child was 7 years old during their annual visit to the research clinics. A Harpenden Stadiometer was used to record standing height to the last complete mm, and a Tanita body fat analyser (model TBF 305) was used to record weight to the nearest 0.1 kg. Body mass index (BMI) was calculated as weight (kg) divided by height (m^2).

- **Maternal education:** this parameter was obtained from a questionnaire completed by the mother at 32 weeks gestation. Education level was determined using a five-point scale including no academic qualifications; vocational training; at least one O-level academic qualification, or equivalent, usually taken at age 16; at least one A-level academic qualification, or equivalent, usually taken at age 18 and a university degree.
- **Maternal social class:** measurement of the household social class was obtained from a questionnaire completed by the mother at 32 weeks gestation. Data regarding occupational title, the mother's job and her partner's job were collected to identify the highest occupational social class. The standard occupational classification (SOC) codes established by the United Kingdom Office of Population Census and Surveys were used to classify social class as I, professional; II, managerial and technical; III, non-manual (i.e. non-manual skilled occupations); III, manual (i.e. manual skilled occupations); IV, part-skilled occupations and V, unskilled occupations.
- **Gestational age:** this parameter was recorded using paediatric and obstetric assessments, the date of the mother's last menstrual period (LMP) and ultrasound measurements. In brief, the date of the LMP was considered if the mother was certain and there were no other clinical suggestions that it was inaccurate. If the LMP date was not certain, then the earliest ultrasound measurement was most likely to be used.
- **Maternal BMI:** maternal height and pre-pregnancy weight were self-reported in a questionnaire completed at 12 weeks gestation, and BMI was calculated accordingly.

- **Pubertal stage:** this parameter was self-reported by the child or their parent in a questionnaire using illustrated drawings following the Tanner staging. Tanner staging describes maturation levels of pubic hair and genital size and is categorised into five stages, with one being the least mature (Marshall and Tanner 1970).

6.3.6 Statistical analysis

Descriptive analysis of continuous variables that were normally distributed was performed using mean and SD, while skewed variables were described using medians and interquartile ranges. Frequencies were used to describe categorical variables (i.e. maternal education, maternal social class and pubertal staging).

Regression coefficient comparison between the outcome measures was performed using z-scores for total fat mass, trunk fat mass, lean fat mass, peripheral fat mass (arms, legs, android and gynoid) and BMI. These variables were not normally distributed and were therefore logged, z-scores were calculated by subtracting the mean from the individual value and then dividing this by the standard deviation.

Three models of regression were used: model 1 included an adjustment for sex and age; model 2 included an adjustment for sex, age, height at the time of the DXA scan, maternal height, maternal BMI, maternal social class, maternal education, gestational age and birth weight and model 3 was similar to model 2 but also included an adjustment for the pubertal stage. Model 1 was also analysed using data only from those for whom complete confounder information was available (model 1*) to evaluate whether changes in effect size were due to missing data bias. Data analysis was conducted using IBM SPSS statistics version 12.0.1.

6.4 Results

6.4.1 Description of ALSPAC sample data

A total of 631 children recruited in the ALSPAC study had complete data available for use in this study. These participants were singletons and had at least one IGF-II measurement and one total fat mass measurement. Of these, 305 (48.3%) were male and 326 (51.7%) were female. As shown in Table 6.1, 366 participants had IGF-II cord blood measurements (mean ± SD: 263.1 ± 83.9 ng/ml), and 365 participants had IGF-II measurements at 61 months old, with a range of 165–695 ng/ml and a mean (±SD) value of 395.7 (±107.5) ng/ml, participants who provided cord blood samples were similar to those collected at 61 months of age.

At the TF3 time point, the mean age of participants was 15.3 ± 0.17 years, the mean height was 169.5 ± 7.9 cm and the average total fat mass measured by DXA was 15.6 ± 9.5 kg. At the F17 time point, the mean age of the participants was 17.6 ± 0.33 years, the average height was 171.7 ± 9.1 cm (range: 149.60–196.30) and the average total fat mass was 17.9 ± 9.9 kg. Further descriptive details of the respondent's fat mass (i.e. trunk, arms, legs, android and gynoid) and lean mass variables are also summarised in Table 6-1.

Table 6-1: Sample size description of IGF-II levels, age, height, body fat mass and lean mass in ALSPAC respondents included in the final analyses at different time points.

Variable	Time point	N	Mean	SD
IGF-II (ng/ml)	Birth (cord blood)	366	263.06	83.9
	61 months	365	395.7	107.47
Age (years)	TF3	552	15.3	0.17
	F17	539	17.6	0.33
Height (cm)	TF3	544	169.5	7.9
	F17	523	171.7	9.1
Total fat mass (kg)[a]	TF3	528	15.6	9.53
	F17	505	17.9	9.94
Trunk fat mass (kg)[a]	TF3	528	7.3	5.07
	F17	508	9.1	5.51
Arm fat mass (kg)[a]	TF3	528	1.2	0.81
	F17	508	1.4	0.83
Leg fat mass (kg)[a]	TF3	528	6.4	3.59
	F17	508	6.6	3.59
Android fat mass (kg)[a]	TF3	528	1.08	0.82
	F17	508	1.29	0.89
Gynoid fat mass (kg)[a]	TF3	528	3.16	1.60
	F17	508	3.58	1.69
Lean mass (kg)[a]	TF3	528	43.42	8.16
	F17	505	46.17	9.96

F17, 17 years; *SD*, standard deviation; *TF3*, 15 years.
[a] median and interquartile ranges.

In addition to age and sex adjustments, maternal factors were also considered in the model 2 regression analysis. The average maternal height was 164.1 ± 6.0 cm (range: 146.4–183.4), and the average maternal BMI was 26.8 ± 5.5 (range: 17.4–52.6). The mean reported length of pregnancy was 39.7 ± 1.4 weeks, and the average birth weight was 3.5 ± 0.5 kg (Table 6-2).

Table 6-2: Description of maternal continuous confounders (i.e. height, BMI, pregnancy length and birth weight) in ALSPAC respondents included in the final analyses.

Variable	N	Mean	SD
Maternal height (cm)	465	164.11	6.00
Maternal BMI	465	26.76	5.48
Pregnancy length (weeks)	631	39.66	1.42
Birth weight (kg)	624	3.49	0.48

BMI, body mass index**;** ***SD,*** standard deviation.

In terms of the maternal education status, most participants had O-level qualifications (38.2%) or A-level qualifications (26.9%). Maternal social class was also considered according to the SOC codes of the Office of Population Census and Surveys. Most mothers had class III skilled non-manual occupations (38.4%) or class II managerial and technical occupations (30.7%). The maternal educational level and social class classification are summarised in Table 6-3.

Table 6-3: Frequency and percentage of maternal education levels and social class of ALSPAC respondents included in the final analysis.

Variable	Classification	Frequency	Percent
Maternal education levels (n = 620)	**CSE**	57	9.2
	Vocational	49	7.9
	O level	241	38.9
	A level	170	27.4
	Degree	103	16.6
Maternal social class (n = 547)	**I**	36	6.6
	II	194	35.5
	III (nonmanual)	242	44.2
	III (manual)	31	5.7
	IV	40	7.3
	V	4	0.7

CSE, certificate of secondary education

In the model 3 regression analysis, further adjustments were required for the pubertal staging as assessed by Tanner staging (described in Section 6.3.5). At the TF3 time point, 222 of the respondents involved in the final analyses had provided pubertal stage information and 409 participants had not. Of these, 120 (19%) were at stage 4 of Tanner's classification and 90 (14.3%) were at stage 5. By F17, the majority had reached stage 5 (83.4%). Details of the pubertal staging frequencies at different time points are described in Table 6-4.

Table 6-4: Frequencies and percentage of pubertal staging categories at TF3 and F17 in the ALSPAC respondents included in the final analyses.

		Frequency		Percent	
Variable	Stage	TF3	F17	TF3	F17
Pubertal staging	Stage 1–3	12	0	5.4	0
	Stage 4	120	61	54.1	16.6
	Stage 5	90	307	40.5	83.4
	Total	**222**	**368**	**100**	**100**

F17, 17 years; TF3, 15 years.

6.4.2 Association between IGF-II levels and total body fat distribution

To overcome the skewed nature of the fat mass data, z-scores were used and calculated as described in Section 6.3.6. Ten-unit block increases in IGF-II was used for analysis, and z-scores of fat mass (total, trunk and peripheral), lean mass and BMI were associated with cord blood IGF-II and IGF-II at 61 months using the three statistical models. Further analysis of model 1 was restricted to participants who had complete data for all variables (model 1*).

Table 6-5 indicates the associations between IGF-II (cord blood and 61 months) and total body fat at TF3 and F17. Cord blood IGF-II was strongly and inversely correlated with total body fat at F17 following adjustment for sex and age (P = 0.017). The

correlation also persisted when analysis model 1* was used, thus ruling out attrition bias; however, this association was attenuated when further adjusting for confounders in models 2 and 3. IGF-II levels at 61 months were weakly and positively correlated with total body fat (P = 0.045) at TF3; however, this correlation was not seen at F17 and did not persist when more confounders were adjusted for or when model 1* restricted analysis was performed. There was no evidence of an association between cord IGF-II and total body fat at TF3.

Table 6-5 : Regression between IGF-II levels (cord blood and 61 months) and total body fat at TF3 and F17 in the ALSPAC cohort.

		IGF-II cord blood				IGF-II 61 months			
		N	**β**	**SE**	**P-value**	**N**	**β**	**SE**	**P-value**
TF3	**Model 1[a]**	303	−0.002	0.006	0.684	309	**0.010**	0.005	**0.045**
	Model 2	204	−0.006	0.007	0.350	202	0.006	0.006	0.261
	Model 3	177	−0.006	0.007	0.408	178	0.006	0.006	0.332
	Model 1*	204	−0.007	0.007	0.281	202	0.005	0.006	0.400
F17	**Model 1**	292	**−0.014**	0.006	**0.017**	286	0.006	0.005	0.253
	Model 2	210	−0.012	0.007	0.067	199	0.005	0.006	0.393
	Model 3	135	−0.009	0.008	0.306	138	0.004	0.007	0.599
	Model 1*	210	**−0.014**	0.007	**0.046**	199	0.004	0.006	0.470

F17, 17 years; *SE*, standard error; *TF3*, 15 years.
[a]Model definitions are provided in Section 6.3.6.

6.4.3 Correlation between IGF-II and trunk fat mass at TF3 and F17.

To further determine the correlation between total body fat and IGF-II, and because central fat deposition is more related to metabolic risk than overall fat (Nguyen-Duy, Nichaman et al. 2003), the association between IGF-II levels (in cord blood and at 61 months) and trunk fat was determined at TF3 and F17. Table 6-6 shows evidence of a

strong negative correlation between IGF-II cord blood levels and trunk fat mass at F17 (P = 0.011) in the minimally-adjusted model and a persisting association between these variables when using the restricted model 1* (P = 0.036). However, this association was attenuated with adjustment for additional confounders. Conversely, there was no evidence of any association between IGF-II cord blood and trunk fat mass when measured at TF3. Furthermore, IGF-II levels at 61 months were not associated with trunk fat at either TF3 or F17.

Table 6-6 : Regression between IGF-II levels (cord blood and 61 months) and trunk body fat at age TF3 and F17 in the ALSPAC cohort.

		IGF-II cord blood				IGF-II 61 months			
		N	**β**	**SE**	**P-value**	**N**	**β**	**SE**	**P-value**
TF3	**Model 1[a]**	303	−0.005	0.006	0.419	309	0.009	0.005	0.056
	Model 2	204	−0.007	0.007	0.297	202	0.005	0.006	0.360
	Model 3	177	−0.007	0.007	0.313	178	0.006	0.061	0.320
	Model 1*	204	−0.008	0.007	0.225	202	0.004	0.006	0.512
F17	**Model 1**	294	**−0.016**	0.006	**0.011**	289	0.006	0.005	0.224
	Model 2	211	−0.013	0.007	0.059	201	0.005	0.006	0.400
	Model 3	136	−0.008	0.009	0.356	140	0.003	0.007	0.703
	Model 1*	211	**−0.015**	0.007	**0.036**	201	0.004	0.006	0.507

F17, 17 years; *SE*, standard error; *TF3*, 15 years.
[a]Model definitions are provided in Section 6.3.6.

6.4.4. Correlation between IGF-II and peripheral fat mass.

6.4.4.1 Correlation between IGF-II and arms and legs fat mass at TF3 and F17.

The relationship between IGF-II and body fat distribution was further analysed by correlating IGF-II levels (cord blood and at 61 months) and peripheral fat in the legs and arms at TF3 and F17 (Table 6-7). The analysis showed strong evidence of an

association between IGF-II at 61 months and arm fat mass at TF3 (P = 0.016); however, the association did not persist when using additional analysis models or when fat mass was measured at F17. In addition, weak evidence of an inverse correlation between cord blood IGF-II and arm fat mass at F17 was seen using the minimally-adjusted model (P = 0.039); however, this association was weakened when adjusting for further confounders and when using model 1*. No evidence of association was seen for IGF-II at 61 months and arm fat mass at F17.

Table 6-7: Regression analysis between IGF-II levels (cord blood and 61 months) and arm fat mass at TF3 and F17 in the ALSPAC cohort.

		IGF-II cord blood				IGF-II 61 months			
		N	**β**	**SE**	**P-value**	**N**	**β**	**SE**	**P-value**
TF3	**Model 1** [a]	303	−0.003	0.005	0.640	309	**0.012**	0.005	**0.016**
	Model 2	204	−0.006	0.006	0.297	202	0.007	0.006	0.237
	Model 3	177	−0.005	0.006	0.391	178	0.006	0.006	0.290
	Model 1*	204	−0.007	0.006	0.236	202	0.005	0.006	0.387
F17	**Model 1**	294	**−0.012**	0.006	**0.039**	289	0.004	0.005	0.376
	Model 2	211	−0.011	0.007	0.091	201	0.004	0.006	0.528
	Model 3	136	−0.009	0.008	0.306	140	0.002	0.007	0.796
	Model 1*	211	−0.013	0.007	0.061	201	0.003	0.006	0.656

F17, 17 years; *SE*, standard error; *TF3*, 15 years.
[a]Model definitions are provided in Section 6.3.6.

Table 6-8 shows evidence of a strong positive correlation between IGF-II at 6 months and leg fat mass at TF3 when minimally adjusted for age and gender (P = 0.011); however, this correlation did not persist when adjusting for further confounders, when the restricted model 1* was used or at F17. Furthermore, no evidence of correlation between cord blood IGF-II and leg fat mass was detected at TF3 or F17.

Table 6-8 : Regression analysis between IGF-II levels (cord blood and 61 months) and leg fat mass in the ALSPAC cohort study.

		IGF-II cord blood				IGF-II 61 months			
		N	**β**	**SE**	**P-value**	**N**	**β**	**SE**	**P-value**
TF3	**Model 1[a]**	303	−0.001	0.006	0.890	309	**0.014**	0.005	**0.011**
	Model 2	204	−0.006	0.006	0.343	202	0.009	0.006	0.138
	Model 3	177	−0.005	0.007	0.439	178	0.007	0.006	0.242
	Model 1*	204	−0.007	0.006	0.291	202	0.007	0.006	0.256
F17	**Model 1**	294	−0.011	0.006	0.053	289	0.005	0.005	0.331
	Model 2	211	−0.009	0.006	0.152	201	0.005	0.006	0.361
	Model 3	136	−0.006	0.008	0.482	140	0.006	0.007	0.426
	Model 1*	211	−0.011	0.007	0.107	201	0.004	0.006	0.479

F17, 17 years; *SE*, standard error; *TF3*, 15 years.
[a]Model definitions are provided in Section 6.3.6.

6.4.4.2 Correlation between IGF-II and android and gynoid fat mass at TF3 and F17

Android and gynoid distribution were previously shown to relate to insulin resistance and metabolic risk (Samsell, Regier et al. 2014), therefore the correlation between IGF-II levels and android and gynoid fat mass was measured at TF3 and F17. Table 6-9 shows that cord blood IGF-II was weakly and negatively correlated with android fat at F17 in model 1 (P = 0.027); however, this correlation did not persist when restricted model 1* was used. Cord blood IGF-II was not correlated with android fat mass at TF3. Examining IGF-II levels at 61 months showed no evidence of correlation with android fat mass at puberty.

Table 6-9 : Regression analysis between IGF-II levels (cord blood and 61 months) and android fat mass at TF3 and F17 in the ALSPAC study.

		IGF-II cord blood				IGF-II 61 months			
		N	**β**	**SE**	**P-value**	**N**	**β**	**SE**	**P-value**
TF3	**Model 1[a]**	303	−0.004	0.006	0.463	309	0.009	0.005	0.088
	Model 2	204	−0.006	0.007	0.374	202	0.004	0.006	0.503
	Model 3	177	−0.006	0.007	0.359	178	0.004	0.006	0.504
	Model 1*	204	−0.008	0.007	0.273	202	0.002	0.006	0.691
F17	**Model 1**	294	**−0.014**	0.006	**0.027**	289	0.004	0.005	0.414
	Model 2	211	−0.010	0.007	0.147	201	0.003	0.006	0.609
	Model 3	136	−0.007	0.009	0.457	140	0.000	0.008	0.959
	Model 1*	211	−0.013	0.007	0.084	201	0.002	0.006	0.746

F17, 17 years; *SE*, standard error; *TF3*, 15 years.
[a]Model definitions are provided in Section 6.3.6.

Table 6-10 shows a similar weak association between cord blood IGF-II and gynoid fat mass at F17 (P = 0.027) when adjusting for age and sex. No correlation was

detected between cord blood IGF-II and gynoid fat mass at TF3, and no correlation was seen between IGF-II at 61 months and gynoid fat mass at either time point.

Table 6-10 : Regression analysis between IGF-II levels (cord blood and 61 months) and gynoid fat mass at TF3 and F17 in the ALSPAC cohort.

		IGF-II cord blood				IGF-II 61 months			
		N	**β**	**SE**	**P-value**	**N**	**β**	**SE**	**P-value**
TF3	**Model 1** [a]	303	−0.001	0.005	0.784	309	0.009	0.005	0.059
	Model 2	204	−0.006	0.007	0.363	202	0.006	0.005	0.284
	Model 3	177	−0.006	0.007	0.395	178	0.005	0.006	0.353
	Model 1*	204	−0.007	0.007	0.287	202	0.005	0.006	0.411
F17	**Model 1**	294	**−0.013**	0.006	**0.027**	289	0.006	0.005	0.227
	Model 2	211	−0.011	0.006	0.091	201	0.006	0.006	0.323
	Model 3	136	−0.008	0.008	0.329	140	0.006	0.007	0.442
	Model 1*	211	−0.013	0.007	0.057	201	0.005	0.006	0.425

F17, 17 years; *SE*, standard error; *TF3*, 15 years.
[a]Model definitions are provided in Section 6.3.6.

6.4.5 Correlation between IGF-II and lean mass fat at TF3 and F17

To further understand the role of IGF-II plasma levels in the regulation of body composition, the correlation between IGF-II levels (cord blood and 61 months) and lean mass at age TF3 and F17 was investigated. No correlation between prepubertal IGF-II and post-pubertal lean mass was observed at TF3 or F17 (Table 6-11).

Table 6-11: Regression analysis between IGF-II levels (cord blood and 61 months) and postpubertal lean mass at TF3 and F17 in the ALSPAC cohort study.

		IGF-II cord blood				IGF-II 61 months			
		N	**β**	**SE**	**P-value**	**N**	**β**	**SE**	**P-value**
TF3	**Model 1[a]**	303	−0.004	0.003	0.126	309	0.002	0.002	0.417
	Model 2	204	−0.001	0.003	0.771	202	0.003	0.003	0.333
	Model 3	177	−0.002	0.003	0.537	178	0.005	0.003	0.112
	Model 1*	204	−0.002	0.003	0.592	202	0.003	0.003	0.325
F17	**Model 1**	292	−0.004	0.003	0.164	286	0.003	0.002	0.135
	Model 2	210	−0.004	0.003	0.133	199	0.002	0.003	0.351
	Model 3	135	−0.007	0.004	0.104	138	0.004	0.003	0.167
	Model 1*	210	−0.004	0.003	0.137	199	0.003	0.003	0.264

F17, 17 years; *SE*, standard error; *TF3*, 15 years.
[a]Model definitions are provided in Section 6.3.6.

6.4.6 Correlation between IGF-II and BMI at TF3 and F17

Table 6-12 illustrates the correlation between IGF-II and BMI at different time points. A strong positive correlation was seen between IGF-II levels at 61 months and BMI at TF3 using model 1; however, this correlation was lost when other models were used and when the analysis was restricted to participants with completed data (model 1*).

There was no correlation between IGF-II cord blood and BMI measured at either TF3 or F17.

Table 6-12: Regression analysis between IGF-II levels (cord blood and 61 months) and postpubertal BMI at TF3 and F17 in the ALSPAC cohort.

		IGF-II cord blood				IGF-II 61 months			
		N	**β**	**SE**	**P-value**	**N**	**β**	**SE**	**P-value**
TF3	**Model 1** [a]	320	−0.008	0.006	0.192	330	**0.014**	0.005	**0.009**
	Model 2	213	−0.007	0.007	0.256	209	0.009	0.006	0.137
	Model 3	184	−0.007	0.007	0.303	182	0.009	0.006	0.158
	Model 1*	213	−0.008	0.007	0.213	209	0.008	0.006	0.246
F17	**Model 1**	307	−0.012	0.006	0.054	304	0.005	0.006	0.333
	Model 2	218	−0.012	0.007	0.070	212	0.005	0.006	0.423
	Model 3	140	−0.010	0.009	0.231	144	0.007	0.007	0.374
	Model 1*	218	−0.014	0.007	0.051	212	0.004	0.006	0.517

F17, 17 years; *SE*, standard error; *TF3*, 15 years.
[a]Model definitions are provided in Section 6.3.6.

6.5 Discussion

The ALSPAC cohort study was used to examine the association between pre-pubertal IGF-II plasma levels (in cord blood and at 61 months old) and body fat composition at puberty measured by DXA scans at TF3 and F17 years. The findings indicate that cord blood IGF-II was strongly and negatively associated with pubertal fat mass at F17, particularly with total body fat and trunk fat mass. In addition, IGF-II levels at 61 months were positively associated with BMI in puberty; however, this association was mainly due to increased peripheral fat mass measured in the legs and arms. These associations were seen when minimal adjustments for confounders (i.e. age and sex) were used.

The correlation between IGF-II levels and body weight has been inconsistently reported in the literature; for example, cross-sectional studies reported that IGF-II levels were lower (Cruickshank, Heald et al. 2001), higher (Frystyk, Vestbo et al. 1995) and similar in obese individuals (Chang, Wu et al. 2002) when compared to normal weight controls. The ALSPAC study previously showed a correlation between IGF-II at 61 months and current fat mass (Ong, Kratzsch et al. 2002), and our observations also indicate an association with fat mass at puberty and seems more related to arm and leg fat mass; however, this association did not persist when adjusting for additional confounders or when using restricted models. The same study also indicated that IGF-II levels in the cord blood were correlated to IGF-II levels at 61 months, thus suggesting that IGF-II levels are genetically regulated (Ong, Kratzsch et al. 2002). Other studies have demonstrated that the methylation status of the IGF-II gene in cord blood was reflected in the circulating plasma levels of IGF-II, and higher circulation levels were associated with higher birth weight in the Newborn Epigenetics

Study cohort (Hoyo, Fortner et al. 2012). However, a contradictory study showed that IGF-I, and not IGF-II, was associated with birth weight (Reece, Wiznitzer et al. 1994). Interestingly, the time point of the IGF-II measurement and the outcome measurement appear to be important; for example, retrospective studies have reported a negative correlation between IGF-II and BMI. The Southern Community Cohort Study identified increasing IGF-II levels with BMI in middle age; however, IGF-II levels were negatively associated with BMI at 21 years of age (Fowke, Matthews et al. 2010). This was also reported in the Boyd Orr Cohort, which correlated IGF levels in old age (mean age: 71 years) with childhood BMI. Similarly, IGF-II correlated positively with BMI and waist–hip ratio at 71 years but showed a negative association with BMI at 6 years of age (Martin, Holly et al. 2006).

Nevertheless, prospective studies showed that low baseline IGF-II levels, may cause higher weight gain after 5 years in middle-aged, non-obese participants. This study reported that for every 100 ng/ml decrease in IGF-II, there is a 0.88 increase in the relative risk for weight gain (Sandhu, Gibson et al. 2003) and that a higher baseline level of IGF-II protected against weight gain. However, this study also indicated that there may be a threshold level for the association between IGF-II and weight. This was reported again in another prospective study in subjects with diabetes, where circulating levels of IGF-II were related to BMI in morbidly obese subjects and these IGF-II levels reduced with weight loss. However, this was not seen in subjects with moderate obesity, in whom IGF-II levels were similar to normal weight individuals (Heald, Kärvestedt et al. 2006).

Whilst IGF-I serum levels having been associated with fat depots, few have examined the relationship between circulating IGF-II levels and fat distribution. IGF-II is known to be a fetal growth factor and has therefore been extensively studied at a genetic level,

and evidence of IGF-II fat depot-specific action was revealed in these genetic studies (Mårin, Kvist et al. 1993). Similar to the results obtained in this study, IGF-II increased expression in young adults due to IGF2/H19 gene methylation showed a fat depot-specific effect, by increasing subcutaneous fat distribution but having no effect on visceral fat distribution (Huang, Galati et al. 2012). This was also seen in individuals with polymorphisms in IGF-II (i.e. Apal AA individuals), who exhibited higher circulating levels of IGF-II that were associated with lower waist–hip measurements (Tchoukalova, Nathanielsz et al. 2009). In post-menopausal women, body fat measured by DXA scan displayed a different association with IGF-II DMR2b methylation according to the location of body fat, preferring a more subcutaneous fat accumulation in comparison to visceral fat (Song, Ernst et al. 2018).

In vitro mammalian studies also showed a fat depot-specific action of IGF-II (Rodríguez, Gaunt et al. 2004), and this was demonstrated using subcutaneous and visceral preadipocytes from children (Chapter 4.4). This was also observed in this study with a positive association between IGF-II levels at 61 months and peripheral fat mass (i.e. arms, legs fat) at TF3 although this was not consistent though all peripheral body fat measurements (android and gynoid fat) and indicate a weak association with BMI at this age. However, the lack of association with lean mass and trunk fat mass indicates that the association with BMI is mainly due to an increase in peripheral fat.

In vitro studies suggest IGF-II may have a regulatory role in myogenesis (Florini, Ewton et al. 1996, Carter, Cosgrove et al. 2009); however, our study indicated no association between circulating levels of IGF-II in early life and lean mass at puberty. This was also reported in later life, as no association was observed between baseline IGF-II and lean mass after 10 years (Bann, Holly et al. 2015).

The strength of this present study is its longitudinal nature as it allows IGF-II levels during early stages of life to be related to post-pubertal measurements. In addition, DXA scans gave detailed descriptions of fat distribution that provided a better idea of metabolic risk than BMI alone. This was clearly seen in this study, as an association between IGF-II and fat mass at T17, but not BMI, was determined. A weakness of the study lies in its lack of generalisability across ethnicities as the vast majority of participants were white (Fraser, Macdonald-Wallis et al. 2012), and evidence has indicated that the correlations between IGF-II levels and weight and BMI are affected by race (Fowke, Matthews et al. 2010). In fact, circulating levels of IGFs are related to genetic factors, and IGF-II levels are more heavily influenced by genes and race, than IGF-I (Harrela, Koistinen et al. 1996, Jernström, Chu et al. 2001).

In conclusion, pre-pubertal circulating levels of IGF-II in early life were negatively associated with trunk fat mass, positively associated with peripheral fat mass (arms and legs) at puberty with adjustment for age and sex, but not associated with lean body mass.

6.6 Conclusion

This study focused on examining the relationship between childhood IGF-II serum levels and body fat distribution at puberty using the ALSPAC cohort study data. We had access to IGF-II levels measured in early life (birth and at 61 months) and DXA scan measurements of fat mass obtained from subcutaneous and visceral body regions at and after puberty (15 and 17 years). This was a logical extension of the laboratory study suggesting a role for IGF-II in fat deposition. The data indicate a fat-depot association between IGF-II and fat mass; a negative association with trunk body fat and a selective positive association with peripheral body fat mass. Furthermore, IGF-II showed a weak positive association with BMI however this seems to be due to increased peripheral body fat as no associations with truncal fat and lean mass was seen. These associations were seen with the adjustments of age and sex and but were attenuated when further confounders were examined.

This work would benefit by replication in additional life-course cohorts.

Chapter 7: General discussion

7.1 General discussion

The IGF system has an established role in regulating many biological processes such as cell survival, growth, differentiation and cell metabolism. IGFs are secreted from most body tissues however, they are not stored within cells but are maintained at high levels in the circulation. Bioavailability is controlled by binding to IGFBPs; around 80-90% of circulating IGF is kept in the vascular compartment by forming a ternary complex with ALS and IGFBP-3, and to a lesser extent IGFBP-5, that modulates tissue availability. The actions of IGFs are mediated through their corresponding receptors which are found in most tissues (Yakar, Liu et al. 1999, Duan, Ren et al. 2010, Werner and LeRoith 2014). An important target tissue for IGF action is adipose tissue, where the receptors are characteristically distributed, allowing a regulatory role for IGF in the growth and expansion of body fat. IGF-I was shown to promote pre-adipocyte proliferation and differentiation in the 3T3-L1 cell line (Humbel 1990) and in primary cultures (Birnie, Ben-Shlomo et al. 2012) and as previously reported, in primary cultures obtained from children (Grohmann, Sabin et al. 2005). IGF-II was recognised for years as a fetal tissue growth regulator, with less known about its postnatal role. One reason is because IGF-II expression is down-regulated postnatally in rodents, whereas humans maintain extremely high levels of IGF-II, with concentrations considerably higher than those of IGF-I (Humbel 1990, Birnie, Ben-Shlomo et al. 2012). A potential role of IGF-II in adipose tissue regulation has emerged because of its association with weight, obesity, and a number of cancers (Feinberg, Koldobskiy et al. 2016). With the increasing epidemic of child and adult obesity and the association of IGF-II genetic variations with body weight and obesity (O'Dell, Miller

et al. 1997, Roth, Schrager et al. 2002, Faienza, Santoro et al. 2010), further understating of the role of IGF-II in adipose tissue is needed.

In this study, we investigated the function of IGF-II as a regulator of adipose tissue growth and examined the fat depot differences in IGF-II actions using paired visceral and subcutaneous fat biopsies obtained from children. The use of paediatric samples was a strength of our study as pre-adipocytes obtained from children display greater viability and have the ability to differentiate for several passages in comparison to adult samples, as previously indicated (Grohmann, Sabin et al. 2005). Furthermore, using paediatric samples minimises the influence of sex-hormone augmentation which accompanies puberty (Sizonenko 1978). In addition, characteristic differences are reported for paediatric adipose tissue compared to that from adults (Rosenbaum, Presta et al. 1991).One of the study aims was to examine fat depot differences in IGF receptor levels, particularly insulin receptor isoforms. Reports have indicated that age can alter the IR-A/IR-B ratio causing an increase in the IR-A: using paediatric samples removes this potential confounder (Serrano, Villar et al. 2005). A minor limitation is that paediatric fat samples are more difficult to obtain due to the existence of fewer operative indications, especially if malignant or inflammatory causes are excluded. As was the case with our sample group, this led to an extension of the sample collection period.

As IGF-II has been described as a potent mitogenic factor (Forbes and Westwood 2010), we investigated the effect of IGF-II on pre-adipocyte proliferation. As speculated, our results showed that IGF-II induced pre-adipocyte proliferation in subcutaneous and visceral pre-adipocytes. Interestingly, in terms of pre-adipocyte differentiation, our data suggest that IGF-II has depot-specific actions in terms of

promoting pre-adipocyte differentiation. IGF-II treatment at physiological concentrations enhances the growth of subcutaneous pre-adipocytes into mature adipocytes and fat deposition, but, in contrast, has a restrictive effect on visceral pre-adipocyte maturation. This effect was associated with an increase in the differentiation markers PPARγ and adiponectin in subcutaneous pre-adipocytes. Interestingly, IGF-II specifically increased PPARγ2 expression, one of the two alternatively spliced forms of PPARγ, indicating that IGF-II alters the splicing preferences of another key adipocyte protein. This may be important as, although PPARγ1 and PPARγ2 are involved in adipogenesis *in vitro*, PPARγ2 is more closely related to nutritional status and the maintenance of insulin sensitivity (Kilroy, Kirk-Ballard et al. 2012). It would be interesting to examine whether proteins involved in alternative splicing are differentially regulated in subcutaneous and visceral adipocytes. Differentiation with IGF-II also increased the levels of the insulin receptor, GLUT4, FASN, and insulin-stimulated glucose uptake in subcutaneous pre-adipocytes with an opposing effect with visceral pre-adipocytes. Recently published supportive evidence also indicates that IGF-II can regulate metabolic *in utero* growth by modulating the genetic expression controlling nutritionally-regulated genes like FASN and PPAR in hepatic cells (Lopez, Zheng et al. 2018).

Potential differences in the action of IGF-II on fat depots has been reported in a limited manner in *in vitro* studies, and only in mammalian experiments (Tchoukalova, Nathanielsz et al. 2009). IGF-II is a genetically highly regulated hormone with restricted imprinted expression and changes in IGF-II gene methylation have been associated with fat depot differences in adipose tissue growth, as seen in younger adults (Huang, Galati et al. 2012) and adults (Rodríguez, Gaunt et al. 2004, Rodríguez, Gaunt et al. 2006). Although those showed that higher IGF-II expression was

associated with less visceral fat accumulation, other studies reported contradictory actions of IGF-II (Roth, Schrager et al. 2002, Martin, Holly et al. 2006). The genetic expression of most imprinted genes is associated with differential methylation of one or multiple regions of the maternal or paternal alleles, with DNA methylation levels influencing the genetic expression of these genes. Interestingly, the methylation status of IGF-II differently methylated regions (DMRs), have been associated with the regulation of body weight and obesity (Murrell, Heeson et al. 2003, Hoyo, Fortner et al. 2012) , and is suggested to be influenced epigenetically. Parental weight peri-conceptually seems to be an important epigenetic effector; obese mothers had reduced IGF-II methylation and increased fetal birth weight (Hoyo, Fortner et al. 2012), while paternal obesity was associated with hypomethylation of the DMRs, leading to altered levels of IGF-II and an increased risk of obesity in the offspring (Soubry, Schildkraut et al. 2013). However, whether the maintenance of normal weight before pregnancy will be protective against the risk of early life obesity in offspring is still to be determined in humans; in animal studies, paternal dietary control modulated the epigenetic expression of IGF-II (Tybl, Shi et al. 2011). Furthermore, in a study of normal weight pregnant women, maternal exercise during pregnancy was linked to a lower infant birth weight and lower IGF-II levels in cord blood (Hopkins, Baldi et al. 2010). Lastly, another recognised postnatal epigenetic influencing factor is breast feeding status, which may modulate the differences in the magnitude of methylation and this has been suggested to relate to the risk associated with being overweight in early life (Perkins, Murphy et al. 2012) although the only randomised trial of breast feeding in Belarus (PROBIT) challenged any effect of breast feeding on obesity (Kramer, Matush et al. 2008).

We considered that differences in IGF receptor distribution might contribute to these IGF-II depot-specific actions (Lefebvre, Laville et al. 1998). As we showed, in agreement with other reports, IGF-IR abundance decreases with pre-adipocyte differentiation (Zizola, Balañá et al. 2002), while insulin receptor expression increases and visceral fat has a higher insulin receptor abundance than subcutaneous fat (Lefebvre, Laville et al. 1998). Insulin receptor isoform distribution—which can markedly alter tissue-specific biological responses to IGF-II—shows a higher distribution of IR-B in tissues that are primarily responsive to insulin action, such as adipocytes, muscle, and liver, whilst IR-A is more abundant in fetal cells and some cancer cells, where mitogenic responses are important (Mosthaf, Grako et al. 1990, Frasca, Pandini et al. 1999); this is consistent with the mainly mitogenic signalling that is reportedly activated by IR-A. Our data indicate that IR-A is the predominant isoform in visceral pre-adipocytes, whilst IR-B predominates in subcutaneous pre-adipocytes. Although the IR-B/IR-A ratio increased with differentiation, IR-A remains significantly higher in visceral adipocytes in comparison to subcutaneous adipocytes, rendering them potentially more IGF-II responsive.

Distribution of the IGF-IIR/M6P differs between the fat depots, which might influence tissue levels of IGF-II. Our data demonstrated that a higher level of IGF-IIR/M6P was found in visceral in comparison to subcutaneous pre-adipocytes. IGF-IIR/M6P plays a role in degrading IGF-II and alterations in IGF-II tissue bio-availability might consequently affect the phosphorylation of other receptors such as the IGF-IR and insulin receptors (Hawkes and Kar 2003). The increased abundance of the IGF-IIR/M6P in visceral cells might indicate lower levels of IGF-II; however, our data show that there was no difference in IGF-II secreted levels between visceral and subcutaneous cells. The IGF-IIR/M6P is not specific to IGF-II alone; the M6P

component could mediate the effects of other ligands, one of which is TGF-β, which is known to inhibit adipogenesis (Choy, Skillington et al. 2000) and could also contribute to the fat depot differences in differentiation that were reported to cause less visceral fat growth.

Fine-tuning the regulation of IGF-II bioavailability is important to control activity and prevent over-exposure, which might be detrimental to health. As previously mentioned, IGFBPs play a crucial role in controlling the amount of free IGFs that are available. IGFBP-3, in particular, is highly related to adipogenesis (Chan, Schedlich et al. 2009) and circulating IGFBP-3 levels have been correlated to body weight in several studies. In younger age groups, IGFBP-3 levels were increased in obese children (Park, Kim et al. 1999) and adolescents and were more closely related to cardiovascular risk (Kong, Choi et al. 2011). In contrast, another study reported that IGFBP-3 levels were decreased in obese adolescents but this was associated with an increase in IGFBP-3 proteolysis (Mohanraj, Kim et al. 2013). Proteolysed IGFBP-3 results in changes in conformation of the motifs responsible for IGF binding, which leads to a lower IGF binding affinity, further explaining the increase in IGF bio-activity related to obesity. The increase in proteolysis was consistent with our findings when cells were subjected to hyperglycaemic conditions. Interestingly, IGFBP-3 has been suggested to be a key factor linking breast cancer advancement with obesity; IGFBP-3 was shown to increase breast cancer progression by T-cell infiltration and enhanced adipose tissue growth in mouse models (Scully, Firth et al. 2016).

Although IGFBP-3 is the most abundant binding protein in the circulation, IGF-II has the greatest affinity for IGFBP-6; however, there are no published data about the role

of IGFBP-6 in adipose tissue development and its influence on IGF-II metabolic regulation is still to be explored.

Additionally, we investigated how acute IGF-II treatment affected mRNA expression of the IGF-IR, the insulin receptor and its isoforms in adipocytes. As predicted, our results indicate that IGF-II exposure has minimal effect on the IGF-IR in adipocytes. However, IGF-II caused down-regulation of total insulin receptor mRNA levels, particularly with respect to IR-A in visceral adipocytes. This down-regulation was associated with reduction in total GLUT4 (insulin sensitive glucose transporter) and glucose uptake into the cell. The reason for IGF-II actions being more profound in visceral adipocytes may include the higher ratio of IR-A in visceral adipocytes in comparison to subcutaneous fat, the higher relative affinity of IGF-II binding to the IR-A (Denley, Bonython et al. 2004), and the fact that the IR-A has a higher internalisation rate than IR-B (Vogt, Carrascosa et al. 1991). It has previously been reported that IGF-II can activate the insulin receptor in human adipocytes at physiological levels (Bäck, Brännmark et al. 2011) and that the ability of IGF-II to modulate glucose uptake is mediated through insulin receptors (Sinha, Buchanan et al. 1990).

The ability of IGF-II to regulate IR-A expression in visceral fat might be of clinical importance, because insulin resistance has been linked to the higher mRNA expression of insulin receptor isoform A (Okabayashi, Maddux et al. 1989). Furthermore, IGF-II has been reported to have an anti-inflammatory effect by reducing TNF-α, associated with obesity and insulin resistance (Morita, Horii et al. 2014). However, the relationship between obesity and IGF-II is far from clear (Chao and D'Amore 2008). A high fat diet has been reported to cause down-regulation of IGF-II expression from

adipocytes and up-regulation of mRNA for the IGF-IIR/M6P, that resulted in a decrease in tissue availability with obesity (Morita, Horii et al. 2014). A conflicting study has indicated that IGF-II secretion and bioavailability are increased in obese individuals, particularly from visceral fat (Gude, Hjortebjerg et al. 2016). The inflammatory cytokines associated with obesity have also been reported to alter the production of IGF-II and IGF binding proteins from the adipose tissue; TNF-α caused a decrease in the production of IGF-II, IGF-I and their IGF binding proteins, whereas IL-1β had no effect on IGF-II but reduced the production of IGF-I. However, whether this obesity-related change in IGF-II is a compensatory mechanism associated with obesity or a cause of obesity requires further investigation and understanding.

Recently, mRNA alternative splicing has been proposed as a mechanism for the pathogenicity of obesity (Wong, Xu et al. 2018). Alternative splicing is an important mechanism for protein generation multiplicity, as genetic splicing is highly regulated (Wang, Liu et al. 2015). Dysregulation of this genetic process was shown to promote metabolic diseases (Kaminska, Hämäläinen et al. 2014) and aging (Holly, Melzer et al. 2013, Latorre and Harries 2017). Insulin receptor mRNA splicing is one of the major receptors involved, with dysregulation noted in diabetes (Huang, Bodkin et al. 1996). The receptor splicing variant was shown to be influenced by weight loss, whether related to nutritional restriction (Kaminska, Hämäläinen et al. 2014) or surgical intervention (Besic, Shi et al. 2015). Insulin receptor alternative splicing can be affected by multiple binding splicing factors, such as the heterogeneous nuclear ribonucleoproteins (hnRNPA1) and serine-arginine rich proteins (SF3A); these factors can bind within exon 11 and promote exon inclusion or exclusion (Talukdar, Sen et al. 2011). In addition to the insulin receptor, other examples of receptor isoforms that are related to obesity include the leptin receptor (OB-R) long, short and soluble

isoforms. The absence of the long isoform has been associated with early onset obesity in a mouse model (Lee, Proenca et al. 1996, Uotani, Bjørbaek et al. 1999), the short leptin isoform is responsible for leptin degradation and has been found to be increased in obesity (Uotani, Bjørbaek et al. 1999), while the soluble isoform of the leptin receptor has been correlated with BMI (Van Dielen, van 't Veer et al. 2002). Other receptor isoforms where genetic mis-splicing is associated with obesity pathology include the lamin A/C gene (LMNA), Lipin-1 and serotonin 2C receptor (5-HTR2c) (Phan and Reue 2005, Miranda, Chacon et al. 2008, Galiveti, Raabe et al. 2014).

This suggests that mRNA alternative splicing can be used as a future therapeutic approach for obesity, by using gene therapy to target and modulate the errors in splicing of these metabolic regulatory genes. Using animals, splice-specific KO mice models have been created, where a particular splice-variant can be overexpressed (Wong, Xu et al. 2018). Furthermore, mRNA alternative splicing was shown to be involved in the regulation of adipose tissue growth, in terms of regulating the processing of brown adipose tissue and thermogenesis, which also makes it an important mechanism for therapeutic obesity intervention (Vernia, Edwards et al. 2016).

Having shown that IGF-II acts as a regulator of adipose tissue biology and growth, we investigated whether IGF-II serum levels could be used as an indicator of future weight and body fat accumulation. In the ALSPAC study, we assessed the relationship between early childhood IGF-II serum levels and pubertal fat accumulation. Interestingly, our data showed that IGF-II is negatively correlated with trunk fat mass and shows a positive association with peripheral fat, in particular on the arms and legs; however, the correlation between IGF-II and peripheral body fat was inconsistent

throughout the peripheral fat measurements (android and gynoid) and was attenuated when confounders other than age and sex were added to the model. Our data indicate that BMI was weakly associated with IGF-II and that the increase in weight was mainly due to peripheral fat increase, as no association with lean mass was observed. Our study was unique in looking for fat accumulation in different body compartments and the use of DXA scans gave more strength and precision to adiposity measurements. Most studies have investigated prenatal IGF-II levels in relation to birth weight, since IGF-II plays an important role in fetal development; the levels of IGF-II correlated positively (Smerieri, Petraroli et al. 2011) or showed no correlation with birth weight (Reece, Wiznitzer et al. 1994). Different results have also been obtained in relation to IGF-II and postnatal obesity: in obese children, low IGF-II levels have been documented, along with lower IGFBP-2 concentrations, particularly in those with insulin resistance and evidence of chronic inflammation (higher expression of TNF-alpha and IL-6) (Street, Smerieri et al. 2013). In contrast, another study showed that IGF-II was significantly higher in obese children and correlated with parameters of insulin sensitivity (Inzaghi, Baldini Ferroli et al. 2017). Nevertheless, the increase in IGF-II associated with obesity might be predicted to correspond to the increase in adipose tissue accompanying obesity. The question of whether IGF-II can be used as a biomarker to predict obesity or insulin-resistant risk later in life has not yet been answered. A more recent report indicated that IGF-II or IGF-I prenatally was not strongly associated with adiposity after birth measured at 1 year of age; however, in that study, they used skinfold thickness as an indicator of obesity, which might not be very accurate. The study further suggested other biomarkers to be better able to predict metabolic health postnatally; higher levels of prenatal adiponectin were correlated to lower beta cell function (HOMA-β) at age 1

year and were positively associated with skinfold thickness measured at age 3 years. In contrast, blood cord insulin levels were positively related to adiposity, and Ghrelin was negatively related to skinfold thickness and BMI (Zhang, Du et al. 2018). Moreover, leptin cord blood levels were negatively related to weight gain in early childhood, as indicated by BMI measurements (Mantzoros, Rifas-Shiman et al. 2009, Boeke, Mantzoros et al. 2013). The identification of obesity biomarkers earlier in life may help to facilitate early preventive approaches e.g. activity and dietary interventions that may positively impact on future obesity risk and its associated metabolic dysfunction (Must and Strauss 1999). This is of particular importance because early onset obesity has been associated with a higher risk of obesity in adulthood (Deshmukh-Taskar, Nicklas et al. 2006).

Taken together, our results indicate that IGF-II might have a role as a physiological regulator of pre-adipocyte growth and metabolism and may play an important role in regulating body fat composition. IGF-II favours more subcutaneous and limited visceral fat growth. Furthermore, our present study suggests that early life levels of IGF-II could be a prognostic biomarker for body fat distribution later in adolescent life. Interestingly, the actions of IGF-II are not only limited to fat, as IGF-II has been previously reported to be a regulator of muscle mass and skeletal muscle cell differentiation (Wilson and Rotwein 2006). IGF-II expression is needed for the transcription factor MyoD promoting the differentiation of fibroblasts to myoblasts (Wilson and Rotwein 2006). Furthermore, the increased expression of IGF-II was found to be associated with a more muscular, leaner phenotype in pigs (Van Laere, Nguyen et al. 2003). This suggests IGF-II may physiologically modulate body composition and cause a reduction of metabolic risk by favouring muscle formation over that of fat.

7.2 Future directions

Our study provides a detailed assessment of the distribution of IGF receptors in subcutaneous and visceral fat samples obtained from children, which is fundamental information for further studies on adipose tissue regulation. As insulin receptor isoforms were shown to be affected by age (Serrano, Villar et al. 2005) and metabolic dysregulation associated with obesity (Sesti, Marini et al. 1991), it will be useful to compare the changes in receptor predominance in subcutaneous and visceral fat samples obtained from obese children. Another avenue for future study may be examining differences by ethnicity: for example, South Asians have a tendency to greater visceral fat accumulation for any given body mass index (Raji, Seely et al. 2001). One might predict that IGF-II may have different effects in South Asians (perhaps a lesser influence on visceral fat) in comparison to other groups. Our model may also prove useful to examine potential interventions that can modify insulin receptor alternative splicing, for example, the anti-diabetic drug Metformin has been shown to influence the mRNA splicing of insulin receptor though AMPK pathway activation and the regulation of specific RNA binding proteins (Laustriat, Gide et al. 2015). Recent reports have also indicated that the natural polyphenolic phytoalexin, Resveratrol regulates hnRNPA1 splicing factor that modulates insulin receptor splicing (Otsuka, Yamamoto et al. 2018). Both of these reagents has been associated with effects on body weight (Golay 2008, Macarulla, Alberdi et al. 2009), therefore it would be interesting to examine how these reagents can manipulate alternate splicing, and the consequential effect on adipocyte biology .

Furthermore, an important aspect of the development of obesity is diet composition; having paediatric subcutaneous and visceral cultures in our laboratory will allow the effect of multiple dietary products that might alter the growth of adipose tissue to be studied. Well-recognised gut microbial metabolites are short-chain fatty acids (SCFAs) which include acetate, propionate acid, and butyrate acid. It has been reported in animal studies that adding SCFAs to high-fat diet-fed rodents improved body weight and was protective against body fat mass increase and diet-induced obesity (Arora, Loo et al. 2012). In addition, adult human studies showed that SCFAs improve glucose homeostasis, insulin sensitivity and blood lipid measurements (Bourdon, Yokoyama et al. 1999) and induced an increase in leptin production (Xiong, Miyamoto et al. 2004). It would be interesting to study the effect of those fatty acids on pre-adipocyte proliferation and differentiation and examine fat depot differences in metabolism. As short-chain fatty acids are a digestive product from fibre consumed in the diet and are believed to influence the regulation of appetite (Frost, Sleeth et al. 2014), an understanding of the effect of SCFAs on childhood fat tissue development would be important to inform if modulating children's daily intake by increasing fibre content, may limit the risk of a future diet-induced obesity.

Chapter 8: Bibliography

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Chapter 9: Appendices

9.1 Study ethical approval

NHS
Health Research Authority

NRES Committee South West - Exeter
Whitefriars
Level 3
Block B
Lewins Mead
Bristol
BS1 2NT

Telephone:
Fax:

04 July 2014

Professor J.P. Hamilton-Shield
Professor of Diabetes & Metabolic Endocrinology
University of Bristol
Bristol Royal Hospital for Children
Upper Maudlin Street
Bristol
BS2 8AE

Dear Professor Hamilton-Shield

Study title:	**Investigating a role for IGFII in adipocyte regulation: differential effects on visceral adipocytes via insulin receptor isoforms.**
REC reference:	**14/SW/0109**
IRAS project ID:	**152062**

Thank you for your letter of 05 June 2014, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information was considered at the meeting of the Committee held on 03 July 2014. A list of the members who were present at the meeting is attached.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to make a request to postpone publication, please contact the REC Manager, Mrs Kirsten Peck, .

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Participant Information Sheet

1. On page 1 of the Participant Information Sheet the sixth word in the first paragraph should be 'participate' and not 'participant'.
2. Under What if we change our mind? – it should read 'You can withdraw from the study at any time without giving a reason and if you do withdraw this will 'not' affect your child's standard of care'.
3. Under What if there is a problem? – the second paragraph should be removed. This paragraph starts 'What arrangements are made … … etc etc'.

You should notify the REC in writing once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers. The REC will acknowledge receipt and provide a final list of the approved documentation for the study, which can be made available to host organisations to facilitate their permission for the study. Failure to provide the final versions to the REC may cause delay in obtaining permissions.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non clinical trials this is not currently mandatory.

If a sponsor wishes to contest the need for registration they should contact Catherine Blewett (catherineblewett@nhs.net), the HRA does not, however, expect exceptions to be made. Guidance on where to register is provided within IRAS.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Covering letter on headed paper		21 May 2014
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only)		07 April 2014
Letters of invitation to participant	2	21 May 2014
Other - CV Dr Alfares		
Participant consent form	1	07 April 2014
Participant consent form [Assent Form]	2	07 April 2014
Participant information sheet (PIS) [Parent]	2	21 May 2014
Participant information sheet (PIS) [5-8 years]	2	21 May 2014
Participant information sheet (PIS) [Under 5 years]	2	21 May 2014
REC Application Form		08 April 2014
Research protocol or project proposal	1	03 March 2014
Summary CV for Chief Investigator (CI)		10 April 2014

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "*After ethical review – guidance for researchers*" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol

- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

14/SW/0109	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project.

Yours sincerely

[redacted]

p.p. Dr Chris Vallance
Acting Chair

Email: nrescommittee.southwest-exeter@nhs.net

Enclosures: *List of names and professions of members who were present at the meeting and those who submitted written comments*

"After ethical review – guidance for researchers" *[SL-AR2]*

Copy to: *Dr Birgit Whitman*
Dr Diana Benton, University Hospitals Bristol NHS Foundation Trust

NRES Committee South West - Exeter

Attendance at Committee meeting on 03 July 2014

Committee Members:

Name	*Profession*	*Present*	*Notes*
Mrs Isobel Brooks	Retired Social Care Manager	Yes	
Professor Malcolm Cowburn	Professor of Applied Social Science	Yes	
Dr Nicole Dorey	Consultant Clinical Oncologist	Yes	
Mr Christoph Lohan	Pharmacist	Yes	
Dr. Roy J. Powell	Research Design Consultant	Yes	
Joan Ramsay	Retired Associate Director of Nursing (Women and Children) Locum Safeguarding Children Nurse	No	
Mr Phil Regan	Minister of Religion	Yes	
Mrs Carol Richardson	Senior Nurse (Retired)	Yes	
Dr Denise Sheehan	Consultant Oncologist	No	
Dr Chris Vallance	Retired chemist (non-research)	Yes	
Mr Royston Van Tromp	Retired Head Teacher	Yes	

Also in attendance:

Name	*Position (or reason for attending)*
Mrs Kirsten Peck	REC Manager
Mr Robert Kemp	Observer

9.2 Letter of invitation to participant

University of BRISTOL

University Hospitals Bristol NHS
NHS Foundation Trust

Invitation to participate in research study

Dear parents,

We are a team of doctors, nurses and scientists from the school of clinical sciences at the University of Bristol.

We are asking if you would consider helping us in our studies on childhood obesity. In this country one in three children in Year 6 at primary school are overweight or obese (boys 34.8%, girls 31.8%) according to *Public Health England* 2012/13. Obese children are at an increased risk of developing various health problems in later life such as Diabetes and heart disease.

Where we accumulate body fat may have greater importance in the development of obesity related health risks than simply the total amount of fat. Although most fat is held under the skin (subcutaneous) we think central (visceral) fat (sometimes called 'a beer belly') is more closely associated with the development of disease.

Our study is exploring why some people as children have a greater tendency to develop obesity problems than others by looking at fat from under the skin and in the tummy and studying why different hormones and nutrients act on these cells in different ways. We would ask if you would consider helping us in our study **investigating a role for Insulin Growth Factor-II in adipocyte regulation.** You are being asked as your child is due to have surgery where the doctors have easy access to fat from under the skin and in the tummy during their routine operation.

If you would like to consider helping us please read the detailed information sheet included with this letter.

Thank you

21/05/2014 version 2

9.3 Participant information sheet (PIS)

9.3.1 Parent Information sheet

University of BRISTOL

University Hospitals Bristol NHS
NHS Foundation Trust

How does the hormone IGF-II affect fat cell development?

(Investigating a role for Insulin Growth Factor-II in adipocyte regulation)

We are inviting you and your child to participate in our research

Before you decide whether to participate in the study, please take the time to read the participant information sheet carefully and discuss it with others if you wish.

What is the purpose of the study?

We would like to invite you and your child to help us in a research study in which we are looking at why some children and not others develop problems with obesity and later diabetes. To do this work we need to look at children's fat cells (adipocytes) that are under the skin (subcutaneous) and inside the abdomen (visceral). We need to look at these fat cells from children aged up to eight years and of all sizes.

We will look at fat that is taken in pea-sized pieces from children. The children we are asking to take part are those having routine operations on their kidneys when skin and abdominal fat can be taken easily due to the nature of the operation i.e. the operation requires access to the tummy

By looking at these fat cells in the laboratory we plan to do tests that will tell us why different hormones and nutrients act on fat cells in different ways that may be important in telling us why some children get heavy and others remain thin.

Why has my child been asked to participate in the study?

Fat cells from adult patients are "more mature" than those derived from children and do not grow well in the laboratory. Fat cells from children are young and full of growth potential making them excellent cells to do this type of study.

What will happen if I agree to take part?

Version 3 09/07/2014

Page 1 of 4

As we are trying to collect tiny samples of fat from children, the best time to do this is at the time of a routine operation. The fat samples we need are very small and at most will be the size of a pea. The surgeon doing your child's operation would take them during your child's operation. Taking these biopsies will not change or affect the operation at all. Only if the surgeon sees the fat during the routine operation will they take a tiny piece for us. The additional time taken during the operation to take these samples will only be one or two minutes.

It will not affect the recovery time, nor the size or healing of the scar and it certainly will not affect your child after the operation. He/she will not 'miss' the bit of fat. The only other difference from normal is that your child will also be measured for height and weight.
So, in summary, we would take a tiny piece of fat during the operation. Your child will be asleep and will not feel anything at all.

How many people are taking part?

We are trying to recruit twenty children for this study and collect two samples from each child

What will happen next?

We will look at how the fat cells from under the skin and in the abdomen grow when given hormones like insulin and compare how they handle sugar and possibly other nutrients. The information that we have about your child's height and weight will give us valuable information on what the cells are doing to control blood sugar levels in different sized children. Once we have treated the fat and collected the adipocytes, any remaining tissue samples will be disposed of like all other clinical material. Nothing will be stored beyond the end of the experiments except the adipocyte cultures. During the work the tissue will be given a number and not stored in the laboratory with any details that may make it recognizable to your child.

Do I have to take part?

No. Taking part is entirely voluntary. If you would prefer not to take part you do not have to give a reason. Your doctor will not be upset and your child's treatment will not be affected. Only the doctors involved in the study will inspect your child's medical records.

Why do you need my written consent?

The dignity, rights, safety and wellbeing of participants must be the primary consideration in any research practice. We can only do research in collaboration with our patients when they are fully informed of what they are consenting to and willingly provide that consent.

Are there any advantages or disadvantages to taking part?

Because this research will help us understand the mechanisms involved in why fat cells from different places represent a different risk of diabetes there will be no actual advantage to your child. It may lead to other children in the future being helped.

Your child will have his/her usual operation in the same way. The tiny pieces of tissue will be taken during the usual procedure and there will be little or minimal risk.

What do I have to do now?

Please ask any questions you may have or ask for time to think if you want. You are under no pressure to agree to take part. You may find that discussing this with family or friends may help you decide whether to take part or not. Please note that this work will have been authorised to proceed by a committee of people specialized in ensuring that all research in children meets approved standards.

If you are happy, then you will be asked to sign a consent form prior to your child having the operation. You will be given a copy of the Information Sheet and a signed consent form to keep.

Confidentiality and data protection

Apart from your child's age, height and weight we will not be collecting any other personal information about your child. All data collected in this study will be maintained and stored in strict accordance with the data protection regulations. All members of the study team will have a duty of confidentiality to you as research participants.

What if we change our mind?

You can withdraw from the study at any time without giving a reason and if you do withdraw this will not affect your child's standard of care.

What if there is a problem?

If you have concerns about any aspect of the way you have been approached or treated during the course of this study you may wish to contact the hospital's Patient support and complaints team on 0117 342 3604 or write to Patient Support & Complaints Team, Trust Headquarters, University Hospitals Bristol, Marlborough Street, Bristol, BS1 3NU

As all participants are NHS patients, indemnity is provided through the NHS schemes. University Hospitals NHS Foundation Trust is responsible for any clinical negligence on the part of its staff.

Where will the results be published and can we see them?

Whilst all results will be anonymised, we would hope to publish data from the study in a peer-reviewed medical or science journal. If you would like a copy of any publications please let us know and we will keep your name and address and ensure copies of all papers are forwarded to you. This usually takes two to three years from the study start.

Who is organising the research?

The principal investigator of this study is Professor Julian Hamilton-Shield and it is being run at the University of Bristol, School of clinical Sciences, IGFs and Metabolic Endocrinology research group (IMEG) in conjunction with the Bristol Biomedical Research Unit in Nutrition. http://www.uhbristol.nhs.uk/research-innovation/bristol-nutrition-bru/ .If you look on that website you can see this study detailed under projects, theme childhood. None of the doctors is being paid to do this study but a doctor, Maiadah Alfares is doing the work for her research thesis paid for by the government of Saudi Arabia.

Who has/will review this study?

All research in the NHS is looked at by an independent group of people called a Research Ethics committee to protect your safety, rights, well-being and dignity. This study has been reviewed and given a favourable opinion by the NRES Committee South West – Exeter.

Thank you for taking the time to read this Information sheet and considering taking part in our study.

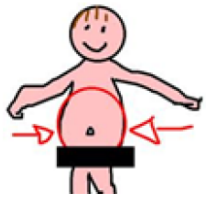
Contact for further information

Professor Hamilton-Shield *(Professor in Diabetes and Metabolic Endocrinology)*
Royal Hospital for Children, Bristol
Phone number: [redacted]

9.3.2 Child information sheet (5-8 years)

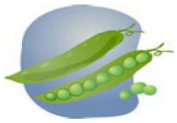
University of BRISTOL

University Hospitals Bristol NHS
NHS Foundation Trust



CHILDREN'S INFORMATION SHEET
(5 – 8 years old)

Why might you be able to help?
Because you are having an operation to make a bit of you better, we would like to ask if we can take two tiny bits of fat at the time: one from under your skin and another one from your tummy. Each would be about the size of a pea. During the operation, while you are asleep, the expert who is doing it would take the tiny bits. It wouldn't hurt as you will be asleep and you wouldn't notice it afterwards. Your body wouldn't need the tiny pieces and you won't feel any different for not having them.



What happens to the fat?
We would compare how your two bits of fat handle sugar and some substances that exist in the blood called hormones.
This will help us to understand why some children get heavy and others stay thin and may help us in the future to help them.

What do you do next?
When you have read this information sheet, think about it for a while and talk about it with your parents. If you have any more questions or want more information then either you or whoever looks after you at home could telephone me using the number at the bottom of this page. Remember that if you don't want to take part it doesn't matter. Your doctor or your family won't be upset and you will have the operation as planned. Also if you decide you want to join in and then change your mind that's OK too. Just tell someone at home and either you or they can telephone me.

Thank you for taking the time to read this.

My name is Dr. Julian Hamilton–Shield
Your parents can contact me at: 0117 342 0183

Version 2 21.5.2014

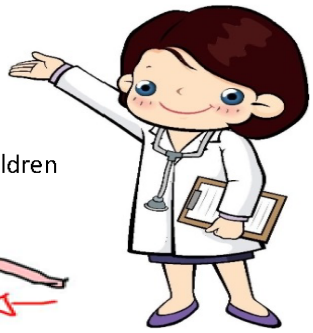
9.3.3 Child information sheet (under 5 years)

University of BRISTOL

University Hospitals Bristol NHS
NHS Foundation Trust

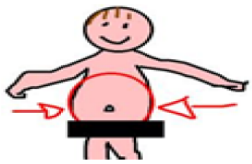
Patient Information Sheet for children under 5 years

Please read this to your child if they are under five years of age and you think they will understand the language



Hello

We would like to ask you to help us find out why some children get heavy and others remain thin.



What do I have to do?

Because you are having an operation to make a bit of you better, we would like to ask if we can take two tiny bits of fat at the time, they would be about the size of a pea. Please don't worry, it won't hurt you as you will be asleep and will not feel anything.

Will this help me?

We can't promise that this will help you but it could help other Children In the future.

Do I have to take part?

If you don't want to take part you don't have to. Nobody will be cross with you. If you say yes, then change your mind later then that's ok too, no one will be upset.

What do I do now?

We know you may have some worries about doing this. Take time to think about if you would like to take part, and ask us or your parents if there is anything you don't understand or you want to know.

version 2 21.5.2014

9.4 Participant consent form

9.4.1 Consent form

University of BRISTOL

University Hospitals Bristol NHS
NHS Foundation Trust

Professor Hamilton-Shield
Royal Hospital for Children, Bristol
Phone number: [redacted]

Patient Identification Number:

CONSENT FORM

Title of Project: **Investigating a role for Insulin Growth Factor-II in adipocyte regulation**

Name of Researcher: **Professor Hamilton-Shield**

Please initial box

1. I confirm that I have read and understand the information sheet dated [09/07/2014] (version 3) for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of any of my medical notes may be looked at by responsible individuals from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐

4. I agree to give fat samples for research in the above project. I understand that giving a sample for this research is voluntary and that I am free to withdraw at any time without giving a reason and without my medical treatment or legal rights being affected. If I decide to stop taking part in the study, I can ask for samples to be destroyed, but I understand that any experimental results already obtained with them will be kept. ☐

5. I agree that the data collected during this study can be stored for future studies, with ethical approval. ☐

6. I agree that the samples collected during this study can be stored for future studies, with ethical approval. ☐

7. I agree to take part in the study.

Name of participant	Date	Signature
Name of person taking consent	Date	Signature

Version 1 07/04/2014

9.4.2 Assent form

University of BRISTOL

University Hospitals Bristol NHS
NHS Foundation Trust

Professor Hamilton-Shield

Royal Hospital for Children, Bristol

Phone number: [redacted]

Patient Identification Number:

ASSENT FORM

(To be completed by the child and their parent/guardian)

Project title: Investigating a role for Insulin Growth Factor-II in adipocyte regulation

Child (or if unable, parent on their behalf) /young person to circle all they agree with:

Has somebody else explained this project to you?	**Yes/No**
Do you understand what this project is about?	**Yes/No**
Have you asked all the questions you want?	**Yes/No**
Have you had your questions answered in a way you understand?	**Yes/No**
Do you understand it's OK to stop taking part at any time?	**Yes/No**
Are you happy to take part?	**Yes/No**

If any answers are "no" or you don't want to take part, don't sign your name!

If you do want to take part, you can write your name below:

Your name:

Date:

The person who explained this project to you needs to sign too:

Print Name:

Sign:

Date:

Thank you for your help.

Version 2 07/04/2014

9.5. ALSPAC study ethical approval

B2840 - Depot specific effect of pre-pubertal IGF-II on post pubertal fat distribution in ALSPAC cohort - 07/03/2017

B number:

B2840

Principal applicant name:

Andy Ness | University of Bristol (Avon)

Co-applicants:

Dr.Maiadah ALfares, Dr Sam Leary, Prof Andy Ness

Title of project:

Depot specific effect of pre-pubertal IGF-II on post pubertal fat distribution in ALSPAC cohort

Proposal summary:

Insulin like growth factor II (IGF-II) is a hormone. IGF-II levels in the human circulation are maintained at high levels throughout life unlike those of rodents. IGF-II has been strongly linked to obesity in genetic studies but its metabolic role is still far from understood. We were able to confirm using fat biopsies from children that IGF-II is a key regulator of fat cell biology. Our results suggest it acts as a buffer to excess visceral fat accumulation in children.

Date proposal received:

Monday, 13 February, 2017

Date proposal approved:

Wednesday, 15 February, 2017

Keywords:

Epidemiology, Obesity, Statistical methods, BMI, Cohort studies - attrition, bias, participant engagement, ethics, Childhood - childcare, childhood adversity, Metabolic - metabolism